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(54) Title: NEISSERIA MENINGITIDIS ANTIGENS AND COMPOSITIONS

(57) Abstract

The invention provides proteins from Neisseria meningitidis, including the amino acid sequences and the corresponding nucleotide sequences. The proteins are predicted to be useful antigens for vaccines and/or diagnostics.

919 (46 kDa)**A) PURIFICATION****M1 919**

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NEISSERIA MENINGITIDIS ANTIGENS AND COMPOSITIONS

This application is a continuation-in-part of the following U.S. Provisional Patent applications, from each of which priority is claimed, and each of which is incorporated by reference in its entirety: 60/083,758 (filed May 1, 1998); 60/094,869 (filed July 31, 1998); 60/098,994 (filed September 2, 1998); 60/099,062 (filed September 2, 1998); 60/103,749 (filed October 9, 1998); 60/103,794 (filed October 9, 1998); 60/103,796 (filed October 9, 1998); and 60/121,528 (filed February 25, 1999).

This invention relates to antigens from the bacterial species: *Neisseria meningitidis* and *Neisseria gonorrhoeae*.

BACKGROUND

Neisseria meningitidis is a non-motile, gram negative diplococcus human pathogen. It colonizes the pharynx, causing meningitis and, occasionally, septicaemia in the absence of meningitis. It is closely related to *N. gonorrhoea*, although one feature that clearly differentiates meningococcus from gonococcus is the presence of a polysaccharide capsule that is present in all pathogenic meningococci.

N. meningitidis causes both endemic and epidemic disease. In the United States the attack rate is 0.6-1 per 100,000 persons per year, and it can be much greater during outbreaks. (see Lieberman *et al.* (1996) Safety and Immunogenicity of a Serogroups A/C *Neisseria meningitidis* Oligosaccharide-Protein Conjugate Vaccine in Young Children. *JAMA* 275(19):1499-1503; Schuchat *et al* (1997) Bacterial Meningitis in the United States in 1995. *N Engl J Med* 337(14):970-976). In developing countries, endemic disease rates are much higher and during epidemics incidence rates can reach 500 cases per 100,000 persons per year. Mortality is extremely high, at 10-20% in the United States, and much higher in developing countries. Following the introduction of the conjugate vaccine against *Haemophilus influenzae*, *N. meningitidis* is the major cause of bacterial meningitis at all ages in the United States (Schuchat *et al* (1997) *supra*).

Based on the organism's capsular polysaccharide, 12 serogroups of *N. meningitidis* have been identified. Group A is the pathogen most often implicated in epidemic disease in sub-Saharan Africa. Serogroups B and C are responsible for the vast majority of cases in the

United States and in most developed countries. Serogroups W135 and Y are responsible for the rest of the cases in the United States and developed countries. The meningococcal vaccine currently in use is a tetravalent polysaccharide vaccine composed of serogroups A, C, Y and W135. Although efficacious in adolescents and adults, it induces a poor immune response and short duration of protection, and cannot be used in infants [eg. Morbidity and Mortality weekly report, Vol.46, No. RR-5 (1997)]. This is because polysaccharides are T-cell independent antigens that induce a weak immune response that cannot be boosted by repeated immunization. Following the success of the vaccination against *H. influenzae*, conjugate vaccines against serogroups A and C have been developed and are at the final stage of clinical testing (Zollinger WD "New and Improved Vaccines Against Meningococcal Disease". In: *New Generation Vaccines, supra*, pp. 469-488; Lieberman *et al* (1996) *supra*; Costantino *et al* (1992) Development and phase I clinical testing of a conjugate vaccine against meningococcus A and C. *Vaccine* 10:691-698).

Meningococcus B (menB) remains a problem, however. This serotype currently is responsible for approximately 50% of total meningitis in the United States, Europe, and South America. The polysaccharide approach cannot be used because the menB capsular polysaccharide is a polymer of $\alpha(2-8)$ -linked *N*-acetyl neuraminic acid that is also present in mammalian tissue. This results in tolerance to the antigen; indeed, if an immune response were elicited, it would be anti-self, and therefore undesirable. In order to avoid induction of autoimmunity and to induce a protective immune response, the capsular polysaccharide has, for instance, been chemically modified substituting the *N*-acetyl groups with *N*-propionyl groups, leaving the specific antigenicity unaltered (Romero & Outschoorn (1994) Current status of Meningococcal group B vaccine candidates: capsular or non-capsular? *Clin Microbiol Rev* 7(4):559-575).

Alternative approaches to menB vaccines have used complex mixtures of outer membrane proteins (OMPs), containing either the OMPs alone, or OMPs enriched in porins, or deleted of the class 4 OMPs that are believed to induce antibodies that block bactericidal activity. This approach produces vaccines that are not well characterized. They are able to protect against the homologous strain, but are not effective at large where there are many antigenic variants of the outer membrane proteins. To overcome the antigenic variability, multivalent vaccines containing up to nine different porins have been constructed (eg. Poolman JT (1992) Development of a meningococcal vaccine. *Infect. Agents Dis.* 4:13-28).

Additional proteins to be used in outer membrane vaccines have been the opa and opc proteins, but none of these approaches have been able to overcome the antigenic variability (eg. Ala'Aldeen & Borriello (1996) The meningococcal transferrin-binding proteins 1 and 2 are both surface exposed and generate bactericidal antibodies capable of killing homologous and heterologous strains. *Vaccine* 14(1):49-53).

A certain amount of sequence data is available for meningococcal and gonococcal genes and proteins (e.g. EP-A-0467714, WO96/29412), but this is by no means complete. Other men B proteins may include those listed in WO 97/28273, WO 96/29412, WO 95/03413, US 5,439,808, and US 5,879,686.

The provision of further sequences could provide an opportunity to identify secreted or surface-exposed proteins that are presumed targets for the immune system and which are not antigenically variable. For instance, some of the identified proteins could be components of efficacious vaccines against meningococcus B, some could be components of vaccines against all meningococcal serotypes, and others could be components of vaccines against all pathogenic *Neisseriae* including *Neisseria meningitidis* or *Neisseria gonorrhoeae*. Those sequences specific to *N. meningitidis* or *N. gonorrhoeae* that are more highly conserved are further preferred sequences.

It is thus an object of the invention is to provide Neisserial DNA sequences which encode proteins that are antigenic or immunogenic.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates the products of protein expression and purification of the predicted ORF 919 as cloned and expressed in *E. coli*.

Fig. 2 illustrates the products of protein expression and purification of the predicted ORF 279 as cloned and expressed in *E. coli*.

Fig. 3 illustrates the products of protein expression and purification of the predicted ORF 576-1 as cloned and expressed in *E. coli*.

Fig. 4 illustrates the products of protein expression and purification of the predicted ORF 519-1 as cloned and expressed in *E. coli*.

Fig. 5 illustrates the products of protein expression and purification of the predicted ORF 121-1 as cloned and expressed in *E. coli*.

Fig. 6 illustrates the products of protein expression and purification of the predicted ORF 128-1 as cloned and expressed in *E. coli*.

Fig. 7 illustrates the products of protein expression and purification of the predicted ORF 206 as cloned and expressed in *E. coli*.

Fig. 8 illustrates the products of protein expression and purification of the predicted ORF 287 as cloned and expressed in *E. coli*.

Fig. 9 illustrates the products of protein expression and purification of the predicted ORF 406 as cloned and expressed in *E. coli*.

Fig. 10 illustrates the hydrophilicity plot, antigenic index and AMPHI regions of the products of protein expression the predicted ORF 919 as cloned and expressed in *E. coli*.

Fig. 11 illustrates the hydrophilicity plot, antigenic index and AMPHI regions of the products of protein expression the predicted ORF 279 as cloned and expressed in *E. coli*.

Fig. 12 illustrates the hydrophilicity plot, antigenic index and AMPHI regions of the products of protein expression the predicted ORF 576-1 as cloned and expressed in *E. coli*.

Fig. 13 illustrates the hydrophilicity plot, antigenic index and AMPHI regions of the products of protein expression the predicted ORF 519-1 as cloned and expressed in *E. coli*.

Fig. 14 illustrates the hydrophilicity plot, antigenic index and AMPHI regions of the products of protein expression the predicted ORF 121-1 as cloned and expressed in *E. coli*.

Fig. 15 illustrates the hydrophilicity plot, antigenic index and AMPHI regions of the products of protein expression the predicted ORF 128-1 as cloned and expressed in *E. coli*.

Fig. 16 illustrates the hydrophilicity plot, antigenic index and AMPHI regions of the products of protein expression the predicted ORF 206 as cloned and expressed in *E. coli*.

Fig. 17 illustrates the hydrophilicity plot, antigenic index and AMPHI regions of the products of protein expression the predicted ORF 287 as cloned and expressed in *E. coli*.

Fig. 18 illustrates the hydrophilicity plot, antigenic index and AMPHI regions of the products of protein expression the predicted ORF 406 as cloned and expressed in *E. coli*.

Fig. 19 shows an alignment comparison of amino acid sequences for ORF 225 for several strains of *Neisseria*. Dark shading indicates regions of homology, and gray shading indicates the conservation of amino acids with similar characteristics. The Figure demonstrates a high degree of conservation among the various strains, further confirming its utility as an antigen for both vaccines and diagnostics.

Fig. 20 shows an alignment comparison of amino acid sequences for ORF 235 for several strains of *Neisseria*. Dark shading indicates regions of homology, and gray shading indicates the conservation of amino acids with similar characteristics. The Figure demonstrates a high degree of conservation among the various strains, further confirming its utility as an antigen for both vaccines and diagnostics.

Fig. 21 shows an alignment comparison of amino acid sequences for ORF 287 for several strains of *Neisseria*. Dark shading indicates regions of homology, and gray shading indicates the conservation of amino acids with similar characteristics. The Figure demonstrates a high degree of conservation among the various strains, further confirming its utility as an antigen for both vaccines and diagnostics.

Fig. 22 shows an alignment comparison of amino acid sequences for ORF 519 for several strains of *Neisseria*. Dark shading indicates regions of homology, and gray shading indicates the conservation of amino acids with similar characteristics. The Figure demonstrates a high degree of conservation among the various strains, further confirming its utility as an antigen for both vaccines and diagnostics.

Fig. 23 shows an alignment comparison of amino acid sequences for ORF 919 for several strains of *Neisseria*. Dark shading indicates regions of homology, and gray shading indicates the conservation of amino acids with similar characteristics. The Figure demonstrates a high degree of conservation among the various strains, further confirming its utility as an antigen for both vaccines and diagnostics.

THE INVENTION

The invention provides proteins comprising the *N. meningitidis* amino acid sequences and *N. gonorrhoeae* amino acid sequences disclosed in the examples.

It also provides proteins comprising sequences homologous (i.e., those having sequence identity) to the *N. meningitidis* amino acid sequences disclosed in the examples. Depending on the particular sequence, the degree of homology (sequence identity) is preferably greater than 50% (eg. 60%, 70%, 80%, 90%, 95%, 99% or more). These proteins include mutants and allelic variants of the sequences disclosed in the examples. Typically, 50% identity or more between two proteins is considered to be an indication of functional equivalence. Identity between proteins is preferably determined by the Smith-Waterman

homology search algorithm as implemented in MPSRCH program (Oxford Molecular) using an affine gap search with parameters: gap penalty 12, gap extension penalty 1.

The invention further provides proteins comprising fragments of the *N. meningitidis* amino acid sequences and *N. gonorrhoeae* amino acid sequences disclosed in the examples. The fragments should comprise at least n consecutive amino acids from the sequences and, depending on the particular sequence, n is 7 or more (eg. 8, 10, 12, 14, 16, 18, 20 or more). Preferably the fragments comprise an epitope from the sequence.

The proteins of the invention can, of course, be prepared by various means (eg. recombinant expression, purification from cell culture, chemical synthesis *etc.*) and in various forms (eg. native, fusions *etc.*). They are preferably prepared in substantially pure or isolated form (*ie.* substantially free from other *N. meningitidis* or *N. gonorrhoeae* host cell proteins)

According to a further aspect, the invention provides antibodies which bind to these proteins. These may be polyclonal or monoclonal and may be produced by any suitable means.

According to a further aspect, the invention provides nucleic acid comprising the *N. meningitidis* nucleotide sequences and *N. gonorrhoeae* nucleotide sequences disclosed in the examples.

According to a further aspect, the invention comprises nucleic acids having sequence identity of greater than 50% (e.g., 60%, 70%, 80%, 90%, 95%, 99% or more) to the nucleic acid sequences herein. Sequence identity is determined as above-discussed.

According to a further aspect, the invention comprises nucleic acid that hybridizes to the sequences provided herein. Conditions for hybridization are set forth herein.

Nucleic acid comprising fragments of these sequences are also provided. These should comprise at least n consecutive nucleotides from the *N. meningitidis* sequences or *N. gonorrhoeae* sequences and depending on the particular sequence, n is 10 or more (eg 12, 14, 15, 18, 20, 25, 30, 35, 40 or more).

According to a further aspect, the invention provides nucleic acid encoding the proteins and protein fragments of the invention.

It should also be appreciated that the invention provides nucleic acid comprising sequences complementary to those described above (eg. for antisense or probing purposes).

Nucleic acid according to the invention can, of course, be prepared in many ways (eg. by chemical synthesis, in part or in whole, from genomic or cDNA libraries, from the

organism itself *etc.*) and can take various forms (eg. single stranded, double stranded, vectors, probes *etc.*).

In addition, the term "nucleic acid" includes DNA and RNA, and also their analogues, such as those containing modified backbones, and also protein nucleic acids (PNA) *etc.*

According to a further aspect, the invention provides vectors comprising nucleotide sequences of the invention (eg. expression vectors) and host cells transformed with such vectors.

According to a further aspect, the invention provides compositions comprising protein, antibody, and/or nucleic acid according to the invention. These compositions may be suitable as vaccines, for instance, or as diagnostic reagents or as immunogenic compositions.

The invention also provides nucleic acid, protein, or antibody according to the invention for use as medicaments (eg. as vaccines) or as diagnostic reagents. It also provides the use of nucleic acid, protein, or antibody according to the invention in the manufacture of (I) a medicament for treating or preventing infection due to Neisserial bacteria (ii) a diagnostic reagent for detecting the presence of Neisserial bacteria or of antibodies raised against Neisserial bacteria or (iii) for raising antibodies. Said Neisserial bacteria may be any species or strain (such as *N. gonorrhoeae*) but are preferably *N. meningitidis*, especially strain B or strain C.

The invention also provides a method of treating a patient, comprising administering to the patient a therapeutically effective amount of nucleic acid, protein, and/or antibody according to the invention.

According to further aspects, the invention provides various processes.

A process for producing proteins of the invention is provided, comprising the step of culturing a host cell according to the invention under conditions which induce protein expression.

A process for detecting polynucleotides of the invention is provided, comprising the steps of: (a) contacting a nucleic probe according to the invention with a biological sample under hybridizing conditions to form duplexes; and (b) detecting said duplexes.

A process for detecting proteins of the invention is provided, comprising the steps of: (a) contacting an antibody according to the invention with a biological sample under conditions suitable for the formation of an antibody-antigen complexes; and (b) detecting said complexes.

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

Methodology - Summary of standard procedures and techniques.

General

This invention provides *Neisseria meningitidis* menB nucleotide sequences, amino acid sequences encoded therein. With these disclosed sequences, nucleic acid probe assays and expression cassettes and vectors can be produced. The expression vectors can be transformed into host cells to produce proteins. The purified or isolated polypeptides (which may also be chemically synthesized) can be used to produce antibodies to detect menB proteins. Also, the host cells or extracts can be utilized for biological assays to isolate agonists or antagonists. In addition, with these sequences one can search to identify open reading frames and identify amino acid sequences. The proteins may also be used in immunogenic compositions, antigenic compositions and as vaccine components.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature e.g., Sambrook *Molecular Cloning: A Laboratory Manual, Second Edition* (1989); *DNA Cloning, Volumes I and ii* (D.N Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed, 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds. 1984); *Transcription and Translation* (B.D. Hames & S.J. Higgins eds. 1984); *Animal Cell Culture* (R.I. Freshney ed. 1986); *Immobilized Cells and Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide to Molecular Cloning* (1984); the *Methods in Enzymology* series (Academic Press, Inc.), especially volumes 154 & 155; *Gene Transfer Vectors for Mammalian Cells* (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory); Mayer and Walker, eds. (1987), *Immunochemical Methods in Cell and Molecular Biology* (Academic Press, London); Scopes, (1987) *Protein Purification: Principles and Practice*, Second Edition (Springer-Verlag, N.Y.), and *Handbook of Experimental Immunology, Volumes I-IV* (D.M. Weir and C.C. Blackwell eds 1986).

Standard abbreviations for nucleotides and amino acids are used in this specification.

All publications, patents, and patent applications cited herein are incorporated in full by reference.

Expression systems

The *Neisseria menB* nucleotide sequences can be expressed in a variety of different expression systems; for example those used with mammalian cells, plant cells, baculoviruses, bacteria, and yeast.

i. Mammalian Systems

Mammalian expression systems are known in the art. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g., structural gene) into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, usually located 25-30 base pairs (bp) upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element, usually located within 100 to 200 bp upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation (Sambrook et al. (1989) "Expression of Cloned Genes in Mammalian Cells." In *Molecular Cloning: A Laboratory Manual*, 2nd ed.).

Mammalian viral genes are often highly expressed and have a broad host range; therefore sequences encoding mammalian viral genes provide particularly useful promoter sequences. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter (Ad MLP), and herpes simplex virus promoter. In addition, sequences derived from non-viral genes, such as the murine metallothionein gene, also provide useful promoter sequences. Expression may be either constitutive or regulated (inducible). Depending on the promoter selected, many promoters may be inducible using known substrates, such as the use of the mouse mammary tumor virus (MMTV) promoter with the glucocorticoid responsive element (GRE) that is induced by glucocorticoid in hormone-responsive transformed cells (see for example, U.S. Patent 5,783,681).

The presence of an enhancer element (enhancer), combined with the promoter elements described above, will usually increase expression levels. An enhancer is a

regulatory DNA sequence that can stimulate transcription up to 1000-fold when linked to homologous or heterologous promoters, with synthesis beginning at the normal RNA start site. Enhancers are also active when they are placed upstream or downstream from the transcription initiation site, in either normal or flipped orientation, or at a distance of more than 1000 nucleotides from the promoter (Maniatis et al. (1987) *Science* 236:1237; Alberts et al. (1989) *Molecular Biology of the Cell*, 2nd ed.). Enhancer elements derived from viruses may be particularly useful, because they usually have a broader host range. Examples include the SV40 early gene enhancer (Dijkema et al (1985) *EMBO J.* 4:761) and the enhancer/promoters derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus (Gorman et al. (1982b) *Proc. Natl. Acad. Sci.* 79:6777) and from human cytomegalovirus (Boshart et al. (1985) *Cell* 41:521). Additionally, some enhancers are regulatable and become active only in the presence of an inducer, such as a hormone or metal ion (Sassone-Corsi and Borelli (1986) *Trends Genet.* 2:215; Maniatis et al. (1987) *Science* 236:1237).

A DNA molecule may be expressed intracellularly in mammalian cells. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide.

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either *in vivo* or *in vitro*. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The adenovirus tripartite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

Usually, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-transcriptional cleavage and polyadenylation (Birnstiel et al. (1985) *Cell* 41:349; Proudfoot and Whitelaw (1988) "Termination and 3' end processing of eukaryotic RNA. In *Transcription and splicing* (ed. B.D. Hames and D.M.

Glover); Proudfoot (1989) *Trends Biochem. Sci.* 14:105). These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator/polyadenylation signals include those derived from SV40 (Sambrook et al (1989) "Expression of cloned genes in cultured mammalian cells." In *Molecular Cloning: A Laboratory Manual*).

Usually, the above described components, comprising a promoter, polyadenylation signal, and transcription termination sequence are put together into expression constructs. Enhancers, introns with functional splice donor and acceptor sites, and leader sequences may also be included in an expression construct, if desired. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as mammalian cells or bacteria. Mammalian replication systems include those derived from animal viruses, which require trans-acting factors to replicate. For example, plasmids containing the replication systems of papovaviruses, such as SV40 (Gluzman (1981) *Cell* 23:175) or polyomavirus, replicate to extremely high copy number in the presence of the appropriate viral T antigen. Additional examples of mammalian replicons include those derived from bovine papillomavirus and Epstein-Barr virus. Additionally, the replicon may have two replication systems, thus allowing it to be maintained, for example, in mammalian cells for expression and in a prokaryotic host for cloning and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2 (Kaufman et al. (1989) *Mol. Cell. Biol.* 9:946) and pHEBO (Shimizu et al. (1986) *Mol. Cell. Biol.* 6:1074).

The transformation procedure used depends upon the host to be transformed. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (e.g., Hep G2), and a number of other cell lines.

ii. Plant Cellular Expression Systems

There are many plant cell culture and whole plant genetic expression systems known in the art. Exemplary plant cellular genetic expression systems include those described in patents, such as: U.S. 5,693,506; US 5,659,122; and US 5,608,143. Additional examples of genetic expression in plant cell culture has been described by Zenk, *Phytochemistry* 30:3861-3863 (1991). Descriptions of plant protein signal peptides may be found in addition to the references described above in Vaulcombe et al., *Mol. Gen. Genet.* 209:33-40 (1987); Chandler et al., *Plant Molecular Biology* 3:407-418 (1984); Rogers, *J. Biol. Chem.* 260:3731-3738 (1985); Rothstein et al., *Gene* 55:353-356 (1987); Whittier et al., *Nucleic Acids Research* 15:2515-2535 (1987); Wirsal et al., *Molecular Microbiology* 3:3-14 (1989); Yu et al., *Gene* 122:247-253 (1992). A description of the regulation of plant gene expression by the phytohormone, gibberellic acid and secreted enzymes induced by gibberellic acid can be found in R.L. Jones and J. MacMillin, *Gibberellins*: in: *Advanced Plant Physiology*, Malcolm B. Wilkins, ed., 1984 Pitman Publishing Limited, London, pp. 21-52. References that describe other metabolically-regulated genes: Sheen, *Plant Cell*, 2:1027-1038(1990); Maas et al., *EMBO J.* 9:3447-3452 (1990); Benkel and Hickey, *Proc. Natl. Acad. Sci.* 84:1337-1339 (1987)

Typically, using techniques known in the art, a desired polynucleotide sequence is inserted into an expression cassette comprising genetic regulatory elements designed for operation in plants. The expression cassette is inserted into a desired expression vector with companion sequences upstream and downstream from the expression cassette suitable for expression in a plant host. The companion sequences will be of plasmid or viral origin and provide necessary characteristics to the vector to permit the vectors to move DNA from an original cloning host, such as bacteria, to the desired plant host. The basic bacterial/plant vector construct will preferably provide a broad host range prokaryote replication origin; a prokaryote selectable marker; and, for *Agrobacterium* transformations, T DNA sequences for *Agrobacterium*-mediated transfer to plant chromosomes. Where the heterologous gene is not readily amenable to detection, the construct will preferably also have a selectable marker gene suitable for determining if a plant cell has been transformed. A general review of suitable markers, for example for the members of the grass family, is found in Wilmink and Dons, 1993, *Plant Mol. Biol. Repr.* 11(2):165-185.

Sequences suitable for permitting integration of the heterologous sequence into the plant genome are also recommended. These might include transposon sequences and the like for homologous recombination as well as Ti sequences which permit random insertion of a heterologous expression cassette into a plant genome. Suitable prokaryote selectable markers include resistance toward antibiotics such as ampicillin or tetracycline. Other DNA sequences encoding additional functions may also be present in the vector, as is known in the art.

The nucleic acid molecules of the subject invention may be included into an expression cassette for expression of the protein(s) of interest. Usually, there will be only one expression cassette, although two or more are feasible. The recombinant expression cassette will contain in addition to the heterologous protein encoding sequence the following elements, a promoter region, plant 5' untranslated sequences, initiation codon depending upon whether or not the structural gene comes equipped with one, and a transcription and translation termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the cassette allow for easy insertion into a pre-existing vector.

A heterologous coding sequence may be for any protein relating to the present invention. The sequence encoding the protein of interest will encode a signal peptide which allows processing and translocation of the protein, as appropriate, and will usually lack any sequence which might result in the binding of the desired protein of the invention to a membrane. Since, for the most part, the transcriptional initiation region will be for a gene which is expressed and translocated during germination, by employing the signal peptide which provides for translocation, one may also provide for translocation of the protein of interest. In this way, the protein(s) of interest will be translocated from the cells in which they are expressed and may be efficiently harvested. Typically secretion in seeds are across the aleurone or scutellar epithelium layer into the endosperm of the seed. While it is not required that the protein be secreted from the cells in which the protein is produced, this facilitates the isolation and purification of the recombinant protein.

Since the ultimate expression of the desired gene product will be in a eucaryotic cell it is desirable to determine whether any portion of the cloned gene contains sequences which will be processed out as introns by the host's splicosome machinery. If so, site-directed mutagenesis of the "intron" region may be conducted to prevent losing a portion of the genetic message as a false intron code, Reed and Maniatis, *Cell* 41:95-105, 1985.

The vector can be microinjected directly into plant cells by use of micropipettes to mechanically transfer the recombinant DNA. Crossway, *Mol. Gen. Genet.*, 202:179-185, 1985. The genetic material may also be transferred into the plant cell by using polyethylene glycol, Krens, et al., *Nature*, 296, 72-74, 1982. Another method of introduction of nucleic acid segments is high velocity ballistic penetration by small particles with the nucleic acid either within the matrix of small beads or particles, or on the surface, Klein, et al., *Nature*, 327, 70-73, 1987 and Knudsen and Muller, 1991, *Planta*, 185:330-336 teaching particle bombardment of barley endosperm to create transgenic barley. Yet another method of introduction would be fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies, Fraley, et al., *Proc. Natl. Acad. Sci. USA*, 79, 1859-1863, 1982.

The vector may also be introduced into the plant cells by electroporation. (Fromm et al., *Proc. Natl Acad. Sci. USA* 82:5824, 1985). In this technique, plant protoplasts are electroporated in the presence of plasmids containing the gene construct. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and form plant callus.

All plants from which protoplasts can be isolated and cultured to give whole regenerated plants can be transformed by the present invention so that whole plants are recovered which contain the transferred gene. It is known that practically all plants can be regenerated from cultured cells or tissues, including but not limited to all major species of sugarcane, sugar beet, cotton, fruit and other trees, legumes and vegetables. Some suitable plants include, for example, species from the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersion*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Cichorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Hererocallis*, *Nemesia*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browaalia*, *Glycine*, *Lolium*, *Zea*, *Triticum*, *Sorghum*, and *Datura*.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts containing copies of the heterologous gene is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced from the protoplast suspension.

These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Shoots and roots normally develop simultaneously. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is fully reproducible and repeatable.

In some plant cell culture systems, the desired protein of the invention may be excreted or alternatively, the protein may be extracted from the whole plant. Where the desired protein of the invention is secreted into the medium, it may be collected. Alternatively, the embryos and embryoless-half seeds or other plant tissue may be mechanically disrupted to release any secreted protein between cells and tissues. The mixture may be suspended in a buffer solution to retrieve soluble proteins. Conventional protein isolation and purification methods will be then used to purify the recombinant protein. Parameters of time, temperature pH, oxygen, and volumes will be adjusted through routine methods to optimize expression and recovery of heterologous protein.

iii. Baculovirus Systems

The polynucleotide encoding the protein can also be inserted into a suitable insect expression vector, and is operably linked to the control elements within that vector. Vector construction employs techniques which are known in the art. Generally, the components of the expression system include a transfer vector, usually a bacterial plasmid, which contains both a fragment of the baculovirus genome, and a convenient restriction site for insertion of the heterologous gene or genes to be expressed; a wild type baculovirus with a sequence homologous to the baculovirus-specific fragment in the transfer vector (this allows for the homologous recombination of the heterologous gene in to the baculovirus genome); and appropriate insect host cells and growth media.

After inserting the DNA sequence encoding the protein into the transfer vector, the vector and the wild type viral genome are transfected into an insect host cell where the vector and viral genome are allowed to recombine. The packaged recombinant virus is expressed and recombinant plaques are identified and purified. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, *inter alia*, Invitrogen, San Diego CA ("MaxBac" kit). These techniques are generally known to

those skilled in the art and fully described in Summers and Smith, *Texas Agricultural Experiment Station Bulletin No. 1555* (1987) (hereinafter "Summers and Smith").

Prior to inserting the DNA sequence encoding the protein into the baculovirus genome, the above described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are usually assembled into an intermediate transplacement construct (transfer vector). This construct may contain a single gene and operably linked regulatory elements; multiple genes, each with its own set of operably linked regulatory elements; or multiple genes, regulated by the same set of regulatory elements. Intermediate transplacement constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as a bacterium. The replicon will have a replication system, thus allowing it to be maintained in a suitable host for cloning and amplification.

Currently, the most commonly used transfer vector for introducing foreign genes into AcNPV is pAc373. Many other vectors, known to those of skill in the art, have also been designed. These include, for example, pVL985 (which alters the polyhedrin start codon from ATG to ATT, and which introduces a BamHI cloning site 32 basepairs downstream from the ATT; see Luckow and Summers, *Virology* (1989) 17:31.

The plasmid usually also contains the polyhedrin polyadenylation signal (Miller et al. (1988) *Ann. Rev. Microbiol.*, 42:177) and a prokaryotic ampicillin-resistance (*amp*) gene and origin of replication for selection and propagation in *E. coli*.

Baculovirus transfer vectors usually contain a baculovirus promoter. A baculovirus promoter is any DNA sequence capable of binding a baculovirus RNA polymerase and initiating the downstream (5' to 3') transcription of a coding sequence (e.g., structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A baculovirus transfer vector may also have a second domain called an enhancer, which, if present, is usually distal to the structural gene. Expression may be either regulated or constitutive.

Structural genes, abundantly transcribed at late times in a viral infection cycle, provide particularly useful promoter sequences. Examples include sequences derived from the gene encoding the viral polyhedron protein, Friesen et al., (1986) "The Regulation of Baculovirus Gene Expression," in: *The Molecular Biology of Baculoviruses* (ed. Walter Doerfler); EPO

Publ. Nos. 127 839 and 155 476; and the gene encoding the p10 protein, Vlak et al., (1988), *J. Gen. Virol.* 69:765.

DNA encoding suitable signal sequences can be derived from genes for secreted insect or baculovirus proteins, such as the baculovirus polyhedrin gene (Carbonell et al. (1988) *Gene*, 73:409). Alternatively, since the signals for mammalian cell posttranslational modifications (such as signal peptide cleavage, proteolytic cleavage, and phosphorylation) appear to be recognized by insect cells, and the signals required for secretion and nuclear accumulation also appear to be conserved between the invertebrate cells and vertebrate cells, leaders of non-insect origin, such as those derived from genes encoding human (α) α -interferon, Maeda et al., (1985), *Nature* 315:592; human gastrin-releasing peptide, Lebacqz-Verheyden et al., (1988), *Molec. Cell. Biol.* 8:3129; human IL-2, Smith et al., (1985) *Proc. Nat'l Acad. Sci. USA*, 82:8404; mouse IL-3, (Miyajima et al., (1987) *Gene* 58:273; and human glucocerebrosidase, Martin et al. (1988) *DNA*, 7:99, can also be used to provide for secretion in insects.

A recombinant polypeptide or polyprotein may be expressed intracellularly or, if it is expressed with the proper regulatory sequences, it can be secreted. Good intracellular expression of nonfused foreign proteins usually requires heterologous genes that ideally have a short leader sequence containing suitable translation initiation signals preceding an ATG start signal. If desired, methionine at the N-terminus may be cleaved from the mature protein by *in vitro* incubation with cyanogen bromide.

Alternatively, recombinant polyproteins or proteins which are not naturally secreted can be secreted from the insect cell by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in insects. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the translocation of the protein into the endoplasmic reticulum.

After insertion of the DNA sequence and/or the gene encoding the expression product precursor of the protein, an insect cell host is co-transformed with the heterologous DNA of the transfer vector and the genomic DNA of wild type baculovirus -- usually by co-transfection. The promoter and transcription termination sequence of the construct will usually comprise a 2-5kb section of the baculovirus genome. Methods for introducing heterologous DNA into the desired site in the baculovirus virus are known in the art. (See

Summers and Smith *supra*; Ju et al. (1987); Smith et al., *Mol. Cell. Biol.* (1983) 3:2156; and Luckow and Summers (1989)). For example, the insertion can be into a gene such as the polyhedrin gene, by homologous double crossover recombination; insertion can also be into a restriction enzyme site engineered into the desired baculovirus gene. Miller et al., (1989), *Bioessays* 4:91. The DNA sequence, when cloned in place of the polyhedrin gene in the expression vector, is flanked both 5' and 3' by polyhedrin-specific sequences and is positioned downstream of the polyhedrin promoter.

The newly formed baculovirus expression vector is subsequently packaged into an infectious recombinant baculovirus. Homologous recombination occurs at low frequency (between about 1% and about 5%); thus, the majority of the virus produced after cotransfection is still wild-type virus. Therefore, a method is necessary to identify recombinant viruses. An advantage of the expression system is a visual screen allowing recombinant viruses to be distinguished. The polyhedrin protein, which is produced by the native virus, is produced at very high levels in the nuclei of infected cells at late times after viral infection. Accumulated polyhedrin protein forms occlusion bodies that also contain embedded particles. These occlusion bodies, up to 15 μm in size, are highly refractile, giving them a bright shiny appearance that is readily visualized under the light microscope. Cells infected with recombinant viruses lack occlusion bodies. To distinguish recombinant virus from wild-type virus, the transfection supernatant is plaqued onto a monolayer of insect cells by techniques known to those skilled in the art. Namely, the plaques are screened under the light microscope for the presence (indicative of wild-type virus) or absence (indicative of recombinant virus) of occlusion bodies. *Current Protocols in Microbiology* Vol. 2 (Ausubel et al. eds) at 16.8 (Supp. 10, 1990); Summers and Smith, *supra*; Miller et al. (1989).

Recombinant baculovirus expression vectors have been developed for infection into several insect cells. For example, recombinant baculoviruses have been developed for, *inter alia*: *Aedes aegypti*, *Autographa californica*, *Bombyx mori*, *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni* (PCT Pub. No. WO 89/046699; Carbonell et al., (1985) *J. Virol.* 56:153; Wright (1986) *Nature* 321:718; Smith et al., (1983) *Mol. Cell. Biol.* 3:2156; and see generally, Fraser, et al. (1989) *In Vitro Cell. Dev. Biol.* 25:225).

Cells and cell culture media are commercially available for both direct and fusion expression of heterologous polypeptides in a baculovirus/expression system; cell culture technology is generally known to those skilled in the art. See, e.g., Summers and Smith *supra*.

The modified insect cells may then be grown in an appropriate nutrient medium, which allows for stable maintenance of the plasmid(s) present in the modified insect host. Where the expression product gene is under inducible control, the host may be grown to high density, and expression induced. Alternatively, where expression is constitutive, the product will be continuously expressed into the medium and the nutrient medium must be continuously circulated, while removing the product of interest and augmenting depleted nutrients. The product may be purified by such techniques as chromatography, e.g., HPLC, affinity chromatography, ion exchange chromatography, etc.; electrophoresis; density gradient centrifugation; solvent extraction, or the like. As appropriate, the product may be further purified, as required, so as to remove substantially any insect proteins which are also secreted in the medium or result from lysis of insect cells, so as to provide a product which is at least substantially free of host debris, e.g., proteins, lipids and polysaccharides.

In order to obtain protein expression, recombinant host cells derived from the transformants are incubated under conditions which allow expression of the recombinant protein encoding sequence. These conditions will vary, dependent upon the host cell selected. However, the conditions are readily ascertainable to those of ordinary skill in the art, based upon what is known in the art.

iv. Bacterial Systems

Bacterial expression techniques are known in the art. A bacterial promoter is any DNA sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A bacterial promoter may also have a second domain called an operator, that may overlap an adjacent RNA polymerase binding site at which RNA synthesis begins. The operator permits negative regulated (inducible) transcription, as a gene repressor protein may bind the operator and thereby inhibit transcription of a specific gene. Constitutive expression may occur in the absence of negative regulatory elements, such as the operator. In addition, positive regulation may be achieved by a gene activator protein binding sequence, which, if present is usually proximal (5') to the RNA polymerase binding sequence. An example of a gene activator protein is the catabolite activator protein (CAP), which helps

initiate transcription of the lac operon in *Escherichia coli* (*E. coli*) (Raibaud *et al.* (1984) *Annu. Rev. Genet.* 18:173). Regulated expression may therefore be either positive or negative, thereby either enhancing or reducing transcription.

Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose (*lac*) (Chang *et al.* (1977) *Nature* 198:1056), and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (*trp*) (Goeddel *et al.* (1980) *Nuc. Acids Res.* 8:4057; Yelverton *et al.* (1981) *Nucl. Acids Res.* 9:731; U.S. Patent 4,738,921; EPO Publ. Nos. 036 776 and 121 775). The beta-lactamase (*bla*) promoter system (Weissmann (1981) "The cloning of interferon and other mistakes." In *Interferon 3* (ed. I. Gresser)), bacteriophage lambda PL (Shimatake *et al.* (1981) *Nature* 292:128) and T5 (U.S. Patent 4,689,406) promoter systems also provide useful promoter sequences.

In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one bacterial or bacteriophage promoter may be joined with the operon sequences of another bacterial or bacteriophage promoter, creating a synthetic hybrid promoter (U.S. Patent 4,551,433). For example, the *tac* promoter is a hybrid *trp-lac* promoter comprised of both *trp* promoter and *lac* operon sequences that is regulated by the *lac* repressor (Amann *et al.* (1983) *Gene* 25:167; de Boer *et al.* (1983) *Proc. Natl. Acad. Sci.* 80:21). Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. A naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of some genes in prokaryotes. The bacteriophage T7 RNA polymerase/promoter system is an example of a coupled promoter system (Studier *et al.* (1986) *J. Mol. Biol.* 189:113; Tabor *et al.* (1985) *Proc Natl. Acad. Sci.* 82:1074). In addition, a hybrid promoter can also be comprised of a bacteriophage promoter and an *E. coli* operator region (EPO Publ. No. 267 851).

In addition to a functioning promoter sequence, an efficient ribosome binding site is also useful for the expression of foreign genes in prokaryotes. In *E. coli*, the ribosome binding site is called the Shine-Dalgarno (SD) sequence and includes an initiation codon (ATG) and a sequence 3-9 nucleotides in length located 3-11 nucleotides upstream of the initiation codon

(Shine *et al.* (1975) *Nature* 254:34). The SD sequence is thought to promote binding of mRNA to the ribosome by the pairing of bases between the SD sequence and the 3' end of *E. coli* 16S rRNA (Steitz *et al.* (1979) "Genetic signals and nucleotide sequences in messenger RNA." In *Biological Regulation and Development: Gene Expression* (ed.-R.F. Goldberger)). To express eukaryotic genes and prokaryotic genes with weak ribosome-binding site, it is often necessary to optimize the distance between the SD sequence and the ATG of the eukaryotic gene (Sambrook *et al.* (1989) "Expression of cloned genes in *Escherichia coli*." In *Molecular Cloning: A Laboratory Manual*).

A DNA molecule may be expressed intracellularly. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide or by either *in vivo* or *in vitro* incubation with a bacterial methionine N-terminal peptidase (EPO Publ. No. 219 237).

Fusion proteins provide an alternative to direct expression. Usually, a DNA sequence encoding the N-terminal portion of an endogenous bacterial protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the bacteriophage lambda cell gene can be linked at the 5' terminus of a foreign gene and expressed in bacteria. The resulting fusion protein preferably retains a site for a processing enzyme (factor Xa) to cleave the bacteriophage protein from the foreign gene (Nagai *et al.* (1984) *Nature* 309:810). Fusion proteins can also be made with sequences from the *lacZ* (Jia *et al.* (1987) *Gene* 60:197), *trpE* (Allen *et al.* (1987) *J. Biotechnol.* 5:93; Makoff *et al.* (1989) *J. Gen. Microbiol.* 135:11), and *Chey* (EPO Publ. No. 324 647) genes. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a processing enzyme (e.g. ubiquitin specific processing-protease) to cleave the ubiquitin from the foreign protein. Through this method, native foreign protein can be isolated (Miller *et al.* (1989) *Bio/Technology* 7:698).

Alternatively, foreign proteins can also be secreted from the cell by creating chimeric DNA molecules that encode a fusion protein comprised of a signal peptide sequence fragment that provides for secretion of the foreign protein in bacteria (U.S. Patent 4,336,336). The

signal sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria). Preferably there are processing sites, which can be cleaved either *in vivo* or *in vitro* encoded between the signal peptide fragment and the foreign gene.

DNA encoding suitable signal sequences can be derived from genes for secreted bacterial proteins, such as the *E. coli* outer membrane protein gene (*ompA*) (Masui *et al.* (1983), in: *Experimental Manipulation of Gene Expression*; Ghrayeb *et al.* (1984) *EMBO J.* 3:2437) and the *E. coli* alkaline phosphatase signal sequence (*phoA*) (Oka *et al.* (1985) *Proc. Natl. Acad. Sci.* 82:7212). As an additional example, the signal sequence of the alpha-amylase gene from various *Bacillus* strains can be used to secrete heterologous proteins from *B. subtilis* (Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:5582; EPO Publ. No. 244 042).

Usually, transcription termination sequences recognized by bacteria are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Transcription termination sequences frequently include DNA sequences of about 50 nucleotides capable of forming stem loop structures that aid in terminating transcription. Examples include transcription termination sequences derived from genes with strong promoters, such as the *trp* gene in *E. coli* as well as other biosynthetic genes.

Usually, the above described components, comprising a promoter, signal sequence (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as bacteria. The replicon will have a replication system, thus allowing it to be maintained in a prokaryotic host either for expression or for cloning and amplification. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably contain at least about 10, and more preferably at least about 20 plasmids. Either a high or low copy number

vector may be selected, depending upon the effect of the vector and the foreign protein on the host.

Alternatively, the expression constructs can be integrated into the bacterial genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to the bacterial chromosome that allows the vector to integrate. Integrations appear to result from recombinations between homologous DNA in the vector and the bacterial chromosome. For example, integrating vectors constructed with DNA from various *Bacillus* strains integrate into the *Bacillus* chromosome (EPO Publ. No. 127 328). Integrating vectors may also be comprised of bacteriophage or transposon sequences.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of bacterial strains that have been transformed. Selectable markers can be expressed in the bacterial host and may include genes which render bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin (neomycin), and tetracycline (Davies *et al.* (1978) *Annu. Rev. Microbiol.* 32:469). Selectable markers may also include biosynthetic genes, such as those in the histidine, tryptophan, and leucine biosynthetic pathways.

Alternatively, some of the above described components can be put together in transformation vectors. Transformation vectors are usually comprised of a selectable marker that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extra-chromosomal replicons or integrating vectors, have been developed for transformation into many bacteria. For example, expression vectors have been developed for, *inter alia*, the following bacteria: *Bacillus subtilis* (Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:5582; EPO Publ. Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541), *Escherichia coli* (Shimatake *et al.* (1981) *Nature* 292:128; Amann *et al.* (1985) *Gene* 40:183; Studier *et al.* (1986) *J. Mol. Biol.* 189:113; EPO Publ. Nos. 036 776, 136 829 and 136 907), *Streptococcus cremoris* (Powell *et al.* (1988) *Appl. Environ. Microbiol.* 54:655); *Streptococcus lividans* (Powell *et al.* (1988) *Appl. Environ. Microbiol.* 54:655), *Streptomyces lividans* (U.S. Patent 4,745,056).

Methods of introducing exogenous DNA into bacterial hosts are well-known in the art, and usually include either the transformation of bacteria treated with CaCl_2 or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by

electroporation. Transformation procedures usually vary with the bacterial species to be transformed. (See e.g., use of *Bacillus*: Masson *et al.* (1989) *FEMS Microbiol. Lett.* 60:273; Palva *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:5582; EPO Publ. Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541; use of *Campylobacter*: Miller *et al.* (1988) *Proc. Natl. Acad. Sci.* 85:856; and Wang *et al.* (1990) *J. Bacteriol.* 172:949; use of *Escherichia coli*: Cohen *et al.* (1973) *Proc. Natl. Acad. Sci.* 69:2110; Dower *et al.* (1988) *Nucleic Acids Res.* 16:6127; Kushner (1978) "An improved method for transformation of *Escherichia coli* with ColE1-derived plasmids. In *Genetic Engineering: Proceedings of the International Symposium on Genetic Engineering* (eds. H.W. Boyer and S. Nicosia); Mandel *et al.* (1970) *J. Mol. Biol.* 53:159; Taketo (1988) *Biochim. Biophys. Acta* 949:318; use of *Lactobacillus*: Chassy *et al.* (1987) *FEMS Microbiol. Lett.* 44:173; use of *Pseudomonas*: Fiedler *et al.* (1988) *Anal. Biochem.* 170:38; use of *Staphylococcus*: Augustin *et al.* (1990) *FEMS Microbiol. Lett.* 66:203; use of *Streptococcus*: Barany *et al.* (1980) *J. Bacteriol.* 144:698; Harlander (1987) "Transformation of *Streptococcus lactis* by electroporation, in: *Streptococcal Genetics* (ed. J. Ferretti and R. Curtiss III); Perry *et al.* (1981) *Infect. Immun.* 32:1295; Powell *et al.* (1988) *Appl. Environ. Microbiol.* 54:655; Somkuti *et al.* (1987) *Proc. 4th Eur. Cong. Biotechnology* 1:412.

v. Yeast Expression

Yeast expression systems are also known to one of ordinary skill in the art. A yeast promoter is any DNA sequence capable of binding yeast RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site (the "TATA Box") and a transcription initiation site. A yeast promoter may also have a second domain called an upstream activator sequence (UAS), which, if present, is usually distal to the structural gene. The UAS permits regulated (inducible) expression. Constitutive expression occurs in the absence of a UAS. Regulated expression may be either positive or negative, thereby either enhancing or reducing transcription.

Yeast is a fermenting organism with an active metabolic pathway, therefore sequences encoding enzymes in the metabolic pathway provide particularly useful promoter sequences.

Examples include alcohol dehydrogenase (ADH) (EPO Publ. No. 284 044), enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate kinase (PyK) (EPO Publ. No. 329 203). The yeast *PHO5* gene, encoding acid phosphatase, also provides useful promoter sequences (Myanohara *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80:1).

In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For example, UAS sequences of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region (U.S. Patent Nos. 4,876,197 and 4,880,734). Other examples of hybrid promoters include promoters which consist of the regulatory sequences of either the *ADH2*, *GAL4*, *GAL10*, OR *PHO5* genes, combined with the transcriptional activation region of a glycolytic enzyme gene such as GAP or PyK (EPO Publ. No. 164 556). Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate transcription. Examples of such promoters include, *inter alia*, (Cohen *et al.* (1980) *Proc. Natl. Acad. Sci. USA* 77:1078; Henikoff *et al.* (1981) *Nature* 283:835; Hollenberg *et al.* (1981) *Curr. Topics Microbiol. Immunol.* 96:119; Hollenberg *et al.* (1979) "The Expression of Bacterial Antibiotic Resistance Genes in the Yeast *Saccharomyces cerevisiae*," in: *Plasmids of Medical, Environmental and Commercial Importance* (eds. K.N. Timmis and A. Puhler); Mercerau-Puigalon *et al.* (1980) *Gene* 11:163; Panthier *et al.* (1980) *Curr. Genet.* 2:109;).

A DNA molecule may be expressed intracellularly in yeast. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by *in vitro* incubation with cyanogen bromide.

Fusion proteins provide an alternative for yeast expression systems, as well as in mammalian, plant, baculovirus, and bacterial expression systems. Usually, a DNA sequence encoding the N-terminal portion of an endogenous yeast protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the yeast or human

superoxide dismutase (SOD) gene, can be linked at the 5' terminus of a foreign gene and expressed in yeast. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. See e.g., EPO Publ. No. 196056. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a processing enzyme (e.g. ubiquitin-specific processing protease) to cleave the ubiquitin from the foreign protein. Through this method, therefore, native foreign protein can be isolated (e.g., WO88/024066).

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provide for secretion in yeast of the foreign protein. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either *in vivo* or *in vitro*. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell.

DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast invertase gene (EPO Publ. No. 012 873; JPO Publ. No. 62:096,086) and the A-factor gene (U.S. Patent 4,588,684). Alternatively, leaders of non-yeast origin, such as an interferon leader, exist that also provide for secretion in yeast (EPO Publ. No. 060 057).

A preferred class of secretion leaders are those that employ a fragment of the yeast alpha-factor gene, which contains both a "pre" signal sequence, and a "pro" region. The types of alpha-factor fragments that can be employed include the full-length pre-pro alpha factor leader (about 83 amino acid residues) as well as truncated alpha-factor leaders (usually about 25 to about 50 amino acid residues) (U.S. Patent Nos. 4,546,083 and 4,870,008; EPO Publ. No. 324 274). Additional leaders employing an alpha-factor leader fragment that provides for secretion include hybrid alpha-factor leaders made with a presequence of a first yeast, but a pro-region from a second yeast alphafactor. (See e.g., PCT Publ. No. WO 89/02463.)

Usually, transcription termination sequences recognized by yeast are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator

sequence and other yeast-recognized termination sequences, such as those coding for glycolytic enzymes.

Usually, the above described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (e.g., plasmids) capable of stable maintenance in a host, such as yeast or bacteria. The replicon may have two replication systems, thus allowing it to be maintained, for example, in yeast for expression and in a prokaryotic host for cloning and amplification. Examples of such yeast-bacteria shuttle vectors include YEp24 (Botstein *et al.* (1979) *Gene* 8:17-24), pCl1 (Brake *et al.* (1984) *Proc. Natl. Acad. Sci USA* 81:4642-4646), and YRp17 (Stinchcomb *et al.* (1982) *J. Mol. Biol.* 158:157). In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably have at least about 10, and more preferably at least about 20. Either a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host. See e.g., Brake *et al.*, *supra*.

Alternatively, the expression constructs can be integrated into the yeast genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to a yeast chromosome that allows the vector to integrate, and preferably contain two homologous sequences flanking the expression construct. Integrations appear to result from recombinations between homologous DNA in the vector and the yeast chromosome (Orr-Weaver *et al.* (1983) *Methods in Enzymol.* 101:228-245). An integrating vector may be directed to a specific locus in yeast by selecting the appropriate homologous sequence for inclusion in the vector. See Orr-Weaver *et al.*, *supra*. One or more expression construct may integrate, possibly affecting levels of recombinant protein produced (Rine *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80:6750). The chromosomal sequences included in the vector can occur either as a single segment in the vector, which results in the integration of the entire vector, or two segments homologous to adjacent segments in the chromosome and flanking the expression construct in the vector, which can result in the stable integration of only the expression construct.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of yeast strains that have been transformed. Selectable markers may include biosynthetic genes that can be expressed in the yeast host, such as *ADE2*, *HIS4*, *LEU2*, *TRP1*, and *ALG7*, and the G418 resistance gene, which confer resistance in yeast cells to tunicamycin and G418, respectively. In addition, a suitable selectable marker may also provide yeast with the ability to grow in the presence of toxic compounds, such as metal. For example, the presence of *CUP1* allows yeast to grow in the presence of copper ions (Butt *et al.* (1987) *Microbiol. Rev.* 51:351).

Alternatively, some of the above described components can be put together into transformation vectors. Transformation vectors are usually comprised of a selectable marker that is either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example, expression vectors and methods of introducing exogenous DNA into yeast hosts have been developed for, *inter alia*, the following yeasts: *Candida albicans* (Kurtz, *et al.* (1986) *Mol. Cell. Biol.* 6:142); *Candida maltosa* (Kunze, *et al.* (1985) *J. Basic Microbiol.* 25:141); *Hansenula polymorpha* (Gleeson, *et al.* (1986) *J. Gen. Microbiol.* 132:3459; Roggenkamp *et al.* (1986) *Mol. Gen. Genet.* 202:302); *Kluyveromyces fragilis* (Das, *et al.* (1984) *J. Bacteriol.* 158:1165); *Kluyveromyces lactis* (De Louvencourt *et al.* (1983) *J. Bacteriol.* 154:737; Van den Berg *et al.* (1990) *Bio/Technology* 8:135); *Pichia guillermondii* (Kunze *et al.* (1985) *J. Basic Microbiol.* 25:141); *Pichia pastoris* (Cregg, *et al.* (1985) *Mol. Cell. Biol.* 5:3376; U.S. Patent Nos. 4,837,148 and 4,929,555); *Saccharomyces cerevisiae* (Hinnen *et al.* (1978) *Proc. Natl. Acad. Sci. USA* 75:1929; Ito *et al.* (1983) *J. Bacteriol.* 153:163); *Schizosaccharomyces pombe* (Beach and Nurse (1981) *Nature* 300:706); and *Yarrowia lipolytica* (Davidow, *et al.* (1985) *Curr. Genet.* 10:380471 Gaillardin, *et al.* (1985) *Curr. Genet.* 10:49).

Methods of introducing exogenous DNA into yeast hosts are well-known in the art, and usually include either the transformation of spheroplasts or of intact yeast cells treated with alkali cations. Transformation procedures usually vary with the yeast species to be transformed. See e.g., [Kurtz *et al.* (1986) *Mol. Cell. Biol.* 6:142; Kunze *et al.* (1985) *J. Basic Microbiol.* 25:141; *Candida*]; [Gleeson *et al.* (1986) *J. Gen. Microbiol.* 132:3459; Roggenkamp *et al.* (1986) *Mol. Gen. Genet.* 202:302; *Hansenula*]; [Das *et al.* (1984) *J.*

Bacteriol. 158:1165; De Louvencourt *et al.* (1983) *J. Bacteriol.* 154:1165; Van den Berg *et al.* (1990) *Bio/Technology* 8:135; Kluyveromyces]; [Cregg *et al.* (1985) *Mol. Cell. Biol.* 5:3376; Kunze *et al.* (1985) *J. Basic Microbiol.* 25:141; U.S. Patent Nos. 4,837,148 and 4,929,555; Pichia]; [Hinnen *et al.* (1978) *Proc. Natl. Acad. Sci. USA* 75:1929; Ito *et al.* (1983) *J. Bacteriol.* 153:163 *Saccharomyces*]; [Beach and Nurse (1981) *Nature* 300:706; *Schizosaccharomyces*]; [Davidow *et al.* (1985) *Curr. Genet.* 10:39; Gaillardin *et al.* (1985) *Curr. Genet.* 10:49; *Yarrowia*].

Definitions

A composition containing X is "substantially free of" Y when at least 85% by weight of the total X+Y in the composition is X. Preferably, X comprises at least about 90% by weight of the total of X+Y in the composition, more preferably at least about 95% or even 99% by weight.

A "conserved" *Neisseria* amino acid fragment or protein is one that is present in a particular *Neisseria* protein in at least x% of *Neisseria*. The value of x may be 50% or more, e.g., 66%, 75%, 80%, 90%, 95% or even 100% (i.e. the amino acid is found in the protein in question in all *Neisseria*). In order to determine whether an amino acid is "conserved" in a particular *Neisseria* protein, it is necessary to compare that amino acid residue in the sequences of the protein in question from a plurality of different *Neisseria* (a reference population). The reference population may include a number of different *Neisseria* species or may include a single species. The reference population may include a number of different serogroups of a particular species or a single serogroup. A preferred reference population consists of the 5 most common *Neisseria* strains.

The term "heterologous" refers to two biological components that are not found together in nature. The components may be host cells, genes, or regulatory regions, such as promoters. Although the heterologous components are not found together in nature, they can function together, as when a promoter heterologous to a gene is operably linked to the gene. Another example is where a *Neisseria* sequence is heterologous to a mouse host cell.

"Epitope" means antigenic determinant, and may elicit a cellular and/or humoral response.

Conditions for "high stringency" are 65 degrees C in 0.1 xSSC 0.5% SDS solution.

An "origin of replication" is a polynucleotide sequence that initiates and regulates replication of polynucleotides, such as an expression vector. The origin of replication behaves as an autonomous unit of polynucleotide replication within a cell, capable of replication under its own control. An origin of replication may be needed for a vector to replicate in a particular host cell. With certain origins of replication, an expression vector can be reproduced at a high copy number in the presence of the appropriate proteins within the cell. Examples of origins are the autonomously replicating sequences, which are effective in yeast; and the viral T-antigen, effective in COS-7 cells.

A "mutant" sequence is defined as a DNA, RNA or amino acid sequence differing from but having homology with the native or disclosed sequence. Depending on the particular sequence, the degree of homology (sequence identity) between the native or disclosed sequence and the mutant sequence is preferably greater than 50% (e.g., 60%, 70%, 80%, 90%, 95%, 99% or more) which is calculated as described above. As used herein, an "allelic variant" of a nucleic acid molecule, or region, for which nucleic acid sequence is provided herein is a nucleic acid molecule, or region, that occurs at essentially the same locus in the genome of another or second isolate, and that, due to natural variation caused by, for example, mutation or recombination, has a similar but not identical nucleic acid sequence. A coding region allelic variant typically encodes a protein having similar activity to that of the protein encoded by the gene to which it is being compared. An allelic variant can also comprise an alteration in the 5' or 3' untranslated regions of the gene, such as in regulatory control regions. (see, for example, U.S. Patent 5,753,235).

Antibodies

As used herein, the term "antibody" refers to a polypeptide or group of polypeptides composed of at least one antibody combining site. An "antibody combining site" is the three-dimensional binding space with an internal surface shape and charge distribution complementary to the features of an epitope of an antigen, which allows a binding of the antibody with the antigen. "Antibody" includes, for example, vertebrate antibodies, hybrid antibodies, chimeric antibodies, humanized antibodies, altered antibodies, univalent antibodies, Fab proteins, and single domain antibodies.

Antibodies against the proteins of the invention are useful for affinity chromatography, immunoassays, and distinguishing/identifying *Neisseria menB* proteins.

Antibodies elicited against the proteins of the present invention bind to antigenic polypeptides or proteins or protein fragments that are present and specifically associated with strains of *Neisseria meningitidis* menB. In some instances, these antigens may be associated with specific strains, such as those antigens specific for the menB strains. The antibodies of the invention may be immobilized to a matrix and utilized in an immunoassay or on an affinity chromatography column, to enable the detection and/or separation of polypeptides, proteins or protein fragments or cells comprising such polypeptides, proteins or protein fragments. Alternatively, such polypeptides, proteins or protein fragments may be immobilized so as to detect antibodies bindably specific thereto.

Antibodies to the proteins of the invention, both polyclonal and monoclonal, may be prepared by conventional methods. In general, the protein is first used to immunize a suitable animal, preferably a mouse, rat, rabbit or goat. Rabbits and goats are preferred for the preparation of polyclonal sera due to the volume of serum obtainable, and the availability of labeled anti-rabbit and anti-goat antibodies. Immunization is generally performed by mixing or emulsifying the protein in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 µg/injection is typically sufficient. Immunization is generally boosted 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vivo* immunization. Polyclonal antisera is obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation (e.g., 1,000g for 10 minutes). About 20-50 ml per bleed may be obtained from rabbits.

Monoclonal antibodies are prepared using the standard method of Kohler & Milstein (*Nature* (1975) 256:495-96), or a modification thereof. Typically, a mouse or rat is immunized as described above. However, rather than bleeding the animal to extract serum, the spleen (and optionally several large lymph nodes) is removed and dissociated into single cells. If desired, the spleen cells may be screened (after removal of nonspecifically adherent cells) by applying a cell suspension to a plate or well coated with the protein antigen. B-cells that express membrane-bound immunoglobulin specific for the antigen bind to the plate, and

are not rinsed away with the rest of the suspension. Resulting B-cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium (e.g., hypoxanthine, aminopterin, thymidine medium, "HAT"). The resulting hybridomas are plated by limiting dilution, and are assayed for the production of antibodies which bind specifically to the immunizing antigen (and which do not bind to unrelated antigens). The selected MAb-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice).

If desired, the antibodies (whether polyclonal or monoclonal) may be labeled using conventional techniques. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ^{32}P and ^{125}I), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase is usually detected by its ability to convert 3,3',5,5'-tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. "Specific binding partner" refers to a protein capable of binding a ligand molecule with high specificity, as for example in the case of an antigen and a monoclonal antibody specific therefor. Other specific binding partners include biotin and avidin or streptavidin, IgG and protein A, and the numerous receptor-ligand couples known in the art. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may serve in several different modes. For example, ^{125}I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as enzyme or as antigen for a MAb. Further, one may combine various labels for desired effect. For example, MAbs and avidin also require labels in the practice of this invention: thus, one might label a MAb with biotin, and detect its presence with avidin labeled with ^{125}I , or with an anti-biotin MAb labeled with HRP. Other permutations and possibilities will be readily apparent to those of ordinary skill in the art, and are considered as equivalents within the scope of the instant invention.

Antigens, immunogens, polypeptides, proteins or protein fragments of the present invention elicit formation of specific binding partner antibodies. These antigens, immunogens, polypeptides, proteins or protein fragments of the present invention comprise immunogenic compositions of the present invention. Such immunogenic compositions may further comprise or include adjuvants, carriers, or other compositions that promote or enhance

or stabilize the antigens, polypeptides, proteins or protein fragments of the present invention. Such adjuvants and carriers will be readily apparent to those of ordinary skill in the art.

Pharmaceutical Compositions

Pharmaceutical compositions can comprise (include) either polypeptides, antibodies, or nucleic acid of the invention. The pharmaceutical compositions will comprise a therapeutically effective amount of either polypeptides, antibodies, or polynucleotides of the claimed invention.

The term "therapeutically effective amount" as used herein refers to an amount of a therapeutic agent to treat, ameliorate, or prevent a desired disease or condition, or to exhibit a detectable therapeutic or preventative effect. The effect can be detected by, for example, chemical markers or antigen levels. Therapeutic effects also include reduction in physical symptoms, such as decreased body temperature, when given to a patient that is febrile. The precise effective amount for a subject will depend upon the subject's size and health, the nature and extent of the condition, and the therapeutics or combination of therapeutics selected for administration. Thus, it is not useful to specify an exact effective amount in advance. However, the effective amount for a given situation can be determined by routine experimentation and is within the judgment of the clinician.

For purposes of the present invention, an effective dose will be from about 0.01 mg/kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of the DNA constructs in the individual to which it is administered.

A pharmaceutical composition can also contain a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent, such as antibodies or a polypeptide, genes, and other therapeutic agents. The term refers to any pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Suitable carriers may be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art.

Pharmaceutically acceptable salts can be used therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of

organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

Pharmaceutically acceptable carriers in therapeutic compositions may contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. Liposomes are included within the definition of a pharmaceutically acceptable carrier.

Delivery Methods

Once formulated, the compositions of the invention can be administered directly to the subject. The subjects to be treated can be animals; in particular, human subjects can be treated.

Direct delivery of the compositions will generally be accomplished by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly or delivered to the interstitial space of a tissue. The compositions can also be administered into a lesion. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal and transcutaneous applications, needles, and gene guns or hyposprays. Dosage treatment may be a single dose schedule or a multiple dose schedule.

Vaccines

Vaccines according to the invention may either be prophylactic (i.e., to prevent infection) or therapeutic (i.e., to treat disease after infection).

Such vaccines comprise immunizing antigen(s) or immunogen(s), immunogenic polypeptide, protein(s) or protein fragments, or nucleic acids (e.g., ribonucleic acid or deoxyribonucleic acid), usually in combination with "pharmaceutically acceptable carriers," which include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or

liposomes), and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents ("adjuvants"). Furthermore, the immunogen or antigen may be conjugated to a bacterial toxoid, such as a toxoid from diphtheria, tetanus, cholera, *H. pylori*, etc. pathogens.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc; (2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (see below) or bacterial cell wall components), such as for example (a) MF59 (PCT Publ. No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (c) RibiTM adjuvant system (RAS), (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL + CWS (DetoxTM); (3) saponin adjuvants, such as StimulonTM (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMs (immunostimulating complexes); (4) Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA); (5) cytokines, such as interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, etc.), interferons (e.g., gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), etc; (6) detoxified mutants of a bacterial ADP-ribosylating toxin such as a cholera toxin (CT), a pertussis toxin (PT), or an *E. coli* heat-labile toxin (LT), particularly LT-K63, LT-R72, CT-S109, PT-K9/G129; see, e.g., WO 93/13302 and WO 92/19265; and (7) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Alum and MF59 are preferred.

As mentioned above, muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

The vaccine compositions comprising immunogenic compositions (e.g., which may include the antigen, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. Alternatively, vaccine compositions comprising immunogenic compositions may comprise an antigen, polypeptide, protein, protein fragment or nucleic acid in a pharmaceutically acceptable carrier.

More specifically, vaccines comprising immunogenic compositions comprise an immunologically effective amount of the immunogenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (e.g., nonhuman primate, primate, *etc.*), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

Typically, the vaccine compositions or immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect, as discussed above under pharmaceutically acceptable carriers.

The immunogenic compositions are conventionally administered parenterally, e.g., by injection, either subcutaneously or intramuscularly. Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories, and transdermal and transcutaneous applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

As an alternative to protein-based vaccines, DNA vaccination may be employed (e.g., Robinson & Torres (1997) *Seminars in Immunology* 9:271-283; Donnelly *et al.* (1997) *Annu Rev Immunol* 15:617-648).

Gene Delivery Vehicles

Gene therapy vehicles for delivery of constructs, including a coding sequence of a therapeutic of the invention, to be delivered to the mammal for expression in the mammal, can be administered either locally or systemically. These constructs can utilize viral or non-viral vector approaches in *in vivo* or *ex vivo* modality. Expression of such coding sequence can be induced using endogenous mammalian or heterologous promoters. Expression of the coding sequence *in vivo* can be either constitutive or regulated.

The invention includes gene delivery vehicles capable of expressing the contemplated nucleic acid sequences. The gene delivery vehicle is preferably a viral vector and, more preferably, a retroviral, adenoviral, adeno-associated viral (AAV), herpes viral, or alphavirus vector. The viral vector can also be an astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, or togavirus viral vector. See generally, Jolly (1994) *Cancer Gene Therapy* 1:51-64; Kimura (1994) *Human Gene Therapy* 5:845-852; Connelly (1995) *Human Gene Therapy* 6:185-193; and Kaplitt (1994) *Nature Genetics* 6:148-153.

Retroviral vectors are well known in the art, including B, C and D type retroviruses, xenotropic retroviruses (for example, NZB-X1, NZB-X2 and NZB9-1 (see O'Neill (1985) *J. Virol.* 53:160) polytropic retroviruses e.g., MCF and MCF-MLV (see Kelly (1983) *J. Virol.* 45:291), spumaviruses and lentiviruses. See RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985.

Portions of the retroviral gene therapy vector may be derived from different retroviruses. For example, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

These recombinant retroviral vectors may be used to generate transduction competent retroviral vector particles by introducing them into appropriate packaging cell lines (see US patent 5,591,624). Retrovirus vectors can be constructed for site-specific integration into host cell DNA by incorporation of a chimeric integrase enzyme into the retroviral particle (see WO96/37626). It is preferable that the recombinant viral vector is a replication defective recombinant virus.

Packaging cell lines suitable for use with the above-described retrovirus vectors are well known in the art, are readily prepared (see WO95/30763 and WO92/05266), and can be used to create producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant vector particles. Preferably, the packaging cell lines are made from human parent cells (e.g., HT1080 cells) or mink parent cell lines, which eliminates inactivation in human serum.

Preferred retroviruses for the construction of retroviral gene therapy vectors include Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis Virus and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe (1976) *J Virol* 19:19-25), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998) and Moloney Murine Leukemia Virus (ATCC No. VR-190). Such retroviruses may be obtained from depositories or collections such as the American Type Culture Collection ("ATCC") in Rockville, Maryland or isolated from known sources using commonly available techniques.

Exemplary known retroviral gene therapy vectors employable in this invention include those described in patent applications GB2200651, EP0415731, EP0345242, EP0334301, WO89/02468; WO89/05349, WO89/09271, WO90/02806, WO90/07936, WO94/03622, WO93/25698, WO93/25234, WO93/11230, WO93/10218, WO91/02805, WO91/02825, WO95/07994, US 5,219,740, US 4,405,712, US 4,861,719, US 4,980,289, US 4,777,127, US 5,591,624. See also Vile (1993) *Cancer Res* 53:3860-3864; Vile (1993) *Cancer Res* 53:962-967; Ram (1993) *Cancer Res* 53 (1993) 83-88; Takamiya (1992) *J Neurosci Res* 33:493-503; Baba (1993) *J Neurosurg* 79:729-735; Mann (1983) *Cell* 33:153; Cane (1984) *Proc Natl Acad Sci* 81:6349; and Miller (1990) *Human Gene Therapy* 1.

Human adenoviral gene therapy vectors are also known in the art and employable in this invention. See, for example, Berkner (1988) *Biotechniques* 6:616 and Rosenfeld (1991) *Science* 252:431, and WO93/07283, WO93/06223, and WO93/07282. Exemplary known adenoviral gene therapy vectors employable in this invention include those described in the above referenced documents and in WO94/12649, WO93/03769, WO93/19191, WO94/28938, WO95/11984, WO95/00655, WO95/27071, WO95/29993, WO95/34671, WO96/05320, WO94/08026, WO94/11506, WO93/06223, WO94/24299, WO95/14102,

WO95/24297, WO95/02697, WO94/28152, WO94/24299, WO95/09241, WO95/25807, WO95/05835, WO94/18922 and WO95/09654. Alternatively, administration of DNA linked to killed adenovirus as described in Curiel (1992) *Hum. Gene Ther.* 3:147-154 may be employed. The gene delivery vehicles of the invention also include adenovirus associated virus (AAV) vectors. Leading and preferred examples of such vectors for use in this invention are the AAV-2 based vectors disclosed in Srivastava, WO93/09239. Most preferred AAV vectors comprise the two AAV inverted terminal repeats in which the native D-sequences are modified by substitution of nucleotides, such that at least 5 native nucleotides and up to 18 native nucleotides, preferably at least 10 native nucleotides up to 18 native nucleotides, most preferably 10 native nucleotides are retained and the remaining nucleotides of the D-sequence are deleted or replaced with non-native nucleotides. The native D-sequences of the AAV inverted terminal repeats are sequences of 20 consecutive nucleotides in each AAV inverted terminal repeat (i.e., there is one sequence at each end) which are not involved in HP formation. The non-native replacement nucleotide may be any nucleotide other than the nucleotide found in the native D-sequence in the same position. Other employable exemplary AAV vectors are pWP-19, pWN-1, both of which are disclosed in Nahreini (1993) *Gene* 124:257-262. Another example of such an AAV vector is psub201 (see Samulski (1987) *J. Virol.* 61:3096). Another exemplary AAV vector is the Double-D ITR vector. Construction of the Double-D ITR vector is disclosed in US Patent 5,478,745. Still other vectors are those disclosed in Carter US Patent 4,797,368 and Muzyczka US Patent 5,139,941, Chartejee US Patent 5,474,935, and Kotin WO94/288157. Yet a further example of an AAV vector employable in this invention is SSV9AFABTKneo, which contains the AFP enhancer and albumin promoter and directs expression predominantly in the liver. Its structure and construction are disclosed in Su (1996) *Human Gene Therapy* 7:463-470. Additional AAV gene therapy vectors are described in US 5,354,678, US 5,173,414, US 5,139,941, and US 5,252,479.

The gene therapy vectors comprising sequences of the invention also include herpes vectors. Leading and preferred examples are herpes simplex virus vectors containing a sequence encoding a thymidine kinase polypeptide such as those disclosed in US 5,288,641 and EP0176170 (Roizman). Additional exemplary herpes simplex virus vectors include HFEM/ICP6-LacZ disclosed in WO95/04139 (Wistar Institute), pHSVlac described in Geller (1988) *Science* 241:1667-1669 and in WO90/09441 and WO92/07945, HSV Us3::pgC-lacZ

described in Fink (1992) *Human Gene Therapy* 3:11-19 and HSV 7134, 2 RH 105 and GAL4 described in EP 0453242 (Breakefield), and those deposited with the ATCC as accession numbers ATCC VR-977 and ATCC VR-260.

Also contemplated are alpha virus gene therapy vectors that can be employed in this invention. Preferred alpha virus vectors are Sindbis viruses vectors. Togaviruses, Semliki Forest virus (ATCC VR-67; ATCC VR-1247), Middleberg virus (ATCC VR-370), Ross River virus (ATCC VR-373; ATCC VR-1246), Venezuelan equine encephalitis virus (ATCC VR923; ATCC VR-1250; ATCC VR-1249; ATCC VR-532), and those described in US patents 5,091,309, 5,217,879, and WO92/10578. More particularly, those alpha virus vectors described in U.S. Serial No. 08/405,627, filed March 15, 1995, WO94/21792, WO92/10578, WO95/07994, US 5,091,309 and US 5,217,879 are employable. Such alpha viruses may be obtained from depositories or collections such as the ATCC in Rockville, Maryland or isolated from known sources using commonly available techniques. Preferably, alphavirus vectors with reduced cytotoxicity are used (see USSN 08/679640).

DNA vector systems such as eukaryotic layered expression systems are also useful for expressing the nucleic acids of the invention. See WO95/07994 for a detailed description of eukaryotic layered expression systems. Preferably, the eukaryotic layered expression systems of the invention are derived from alphavirus vectors and most preferably from Sindbis viral vectors.

Other viral vectors suitable for use in the present invention include those derived from poliovirus, for example ATCC VR-58 and those described in Evans, *Nature* 339 (1989) 385 and Sabin (1973) *J. Biol. Standardization* 1:115; rhinovirus, for example ATCC VR-1110 and those described in Arnold (1990) *J Cell Biochem* L401; pox viruses such as canary pox virus or vaccinia virus, for example ATCC VR-111 and ATCC VR-2010 and those described in Fisher-Hoch (1989) *Proc Natl Acad Sci* 86:317; Flexner (1989) *Ann NY Acad Sci* 569:86, Flexner (1990) *Vaccine* 8:17; in US 4,603,112 and US 4,769,330 and WO89/01973; SV40 virus, for example ATCC VR-305 and those described in Mulligan (1979) *Nature* 277:108 and Madzak (1992) *J Gen Virol* 73:1533; influenza virus, for example ATCC VR-797 and recombinant influenza viruses made employing reverse genetics techniques as described in US 5,166,057 and in Enami (1990) *Proc Natl Acad Sci* 87:3802-3805; Enami & Palese (1991) *J Virol* 65:2711-2713 and Luytjes (1989) *Cell* 59:110, (see also McMichael (1983) *NEJ Med* 309:13, and Yap (1978) *Nature* 273:238 and *Nature* (1979) 277:108); human

immunodeficiency virus as described in EP-0386882 and in Buchschacher (1992) *J. Virol.* 66:2731; measles virus, for example ATCC VR-67 and VR-1247 and those described in EP-0440219; Aura virus, for example ATCC VR-368; Bebaru virus, for example ATCC VR-600 and ATCC VR-1240; Cabassou virus, for example ATCC VR-922; Chikungunya virus, for example ATCC VR-64 and ATCC VR-1241; Fort Morgan Virus, for example ATCC VR-924; Getah virus, for example ATCC VR-369 and ATCC VR-1243; Kyzylagach virus, for example ATCC VR-927; Mayaro virus, for example ATCC VR-66; Mucambo virus, for example ATCC VR-580 and ATCC VR-1244; Ndumu virus, for example ATCC VR-371; Pixuna virus, for example ATCC VR-372 and ATCC VR-1245; Tonate virus, for example ATCC VR-925; Trinita virus, for example ATCC VR-469; Una virus, for example ATCC VR-374; Whataroa virus, for example ATCC VR-926; Y-62-33 virus, for example ATCC VR-375; O'Nyong virus, Eastern encephalitis virus, for example ATCC VR-65 and ATCC VR-1242; Western encephalitis virus, for example ATCC VR-70, ATCC VR-1251, ATCC VR-622 and ATCC VR-1252; and coronavirus, for example ATCC VR-740 and those described in Hamre (1966) *Proc Soc Exp Biol Med* 121:190.

Delivery of the compositions of this invention into cells is not limited to the above mentioned viral vectors. Other delivery methods and media may be employed such as, for example, nucleic acid expression vectors, polycationic condensed DNA linked or unlinked to killed adenovirus alone, for example see US Serial No. 08/366,787, filed December 30, 1994 and Curiel (1992) *Hum Gene Ther* 3:147-154 ligand linked DNA, for example see Wu (1989) *J Biol Chem* 264:16985-16987, eucaryotic cell delivery vehicles cells, for example see US Serial No.08/240,030, filed May 9, 1994, and US Serial No. 08/404,796, deposition of photopolymerized hydrogel materials, hand-held gene transfer particle gun, as described in US Patent 5,149,655, ionizing radiation as described in US5,206,152 and in WO92/11033, nucleic charge neutralization or fusion with cell membranes. Additional approaches are described in Philip (1994) *Mol Cell Biol* 14:2411-2418 and in Woffendin (1994) *Proc Natl Acad Sci* 91:1581-1585.

Particle mediated gene transfer may be employed, for example see US Serial No. 60/023,867. Briefly, the sequence can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, as described in Wu &

Wu (1987) *J. Biol. Chem.* 262:4429-4432, insulin as described in Hucked (1990) *Biochem Pharmacol* 40:253-263, galactose as described in Plank (1992) *Bioconjugate Chem* 3:533-539, lactose or transferrin.

Naked DNA may also be employed to transform a host cell. Exemplary naked DNA introduction methods are described in WO 90/11092 and US 5,580,859. Uptake efficiency may be improved using biodegradable latex beads. DNA coated latex beads are efficiently transported into cells after endocytosis initiation by the beads. The method may be improved further by treatment of the beads to increase hydrophobicity and thereby facilitate disruption of the endosome and release of the DNA into the cytoplasm.

Liposomes that can act as gene delivery vehicles are described in U.S. 5,422,120, WO95/13796, WO94/23697, WO91/14445 and EP-524,968. As described in USSN. 60/023,867, on non-viral delivery, the nucleic acid sequences encoding a polypeptide can be inserted into conventional vectors that contain conventional control sequences for high level expression, and then be incubated with synthetic gene transfer molecules such as polymeric DNA-binding cations like polylysine, protamine, and albumin, linked to cell targeting ligands such as asialoorosomucoid, insulin, galactose, lactose, or transferrin. Other delivery systems include the use of liposomes to encapsulate DNA comprising the gene under the control of a variety of tissue-specific or ubiquitously-active promoters. Further non-viral delivery suitable for use includes mechanical delivery systems such as the approach described in Woffendin *et al* (1994) *Proc. Natl. Acad. Sci. USA* 91(24):11581-11585. Moreover, the coding sequence and the product of expression of such can be delivered through deposition of photopolymerized hydrogel materials. Other conventional methods for gene delivery that can be used for delivery of the coding sequence include, for example, use of hand-held gene transfer particle gun, as described in U.S. 5,149,655; use of ionizing radiation for activating transferred gene, as described in U.S. 5,206,152 and WO92/11033.

Exemplary liposome and polycationic gene delivery vehicles are those described in US 5,422,120 and 4,762,915; in WO 95/13796; WO94/23697; and WO91/14445; in EP-0524968; and in Stryer, *Biochemistry*, pages 236-240 (1975) W.H. Freeman, San Francisco; Szoka (1980) *Biochem Biophys Acta* 600:1; Bayer (1979) *Biochem Biophys Acta* 550:464; Rivnay (1987) *Meth Enzymol* 149:119; Wang (1987) *Proc Natl Acad Sci* 84:7851; Plant (1989) *Anal Biochem* 176:420.

A polynucleotide composition can comprises therapeutically effective amount of a gene therapy vehicle, as the term is defined above. For purposes of the present invention, an effective dose will be from about 0.01 mg/ kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of the DNA constructs in the individual to which it is administered.

Delivery Methods

Once formulated, the polynucleotide compositions of the invention can be administered (1) directly to the subject; (2) delivered *ex vivo*, to cells derived from the subject; or (3) *in vitro* for expression of recombinant proteins. The subjects to be treated can be mammals or birds. Also, human subjects can be treated.

Direct delivery of the compositions will generally be accomplished by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly or delivered to the interstitial space of a tissue. The compositions can also be administered into a tumor or lesion. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal applications, needles, and gene guns or hyposprays. Dosage treatment may be a single dose schedule or a multiple dose schedule.

Methods for the *ex vivo* delivery and reimplantation of transformed cells into a subject are known in the art and described in *eg.* WO93/14778. Examples of cells useful in *ex vivo* applications include, for example, stem cells, particularly hematopoietic, lymph cells, macrophages, dendritic cells, or tumor cells.

Generally, delivery of nucleic acids for both *ex vivo* and *in vitro* applications can be accomplished by the following procedures, for example, dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei, all well known in the art.

Polynucleotide and polypeptide pharmaceutical compositions

In addition to the pharmaceutically acceptable carriers and salts described above, the following additional agents can be used with polynucleotide and/or polypeptide compositions.

A. Polypeptides

One example are polypeptides which include, without limitation: asialoglycoprotein (ASOR); transferrin; asialoglycoproteins; antibodies; antibody fragments; ferritin; interleukins; interferons, granulocyte, macrophage colony stimulating factor (GM-CSF),

granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), stem cell factor and erythropoietin. Viral antigens, such as envelope proteins, can also be used. Also, proteins from other invasive organisms, such as the 17 amino acid peptide from the circumsporozoite protein of plasmodium falciparum known as RII.

B.Hormones, Vitamins, Etc.

Other groups that can be included are, for example: hormones, steroids, androgens, estrogens, thyroid hormone, or vitamins, folic acid.

C.Polyalkylenes, Polysaccharides, etc.

Also, polyalkylene glycol can be included with the desired polynucleotides or polypeptides. In a preferred embodiment, the polyalkylene glycol is polyethylene glycol. In addition, mono-, di-, or polysaccharides can be included. In a preferred embodiment of this aspect, the polysaccharide is dextran or DEAE-dextran. Also, chitosan and poly(lactide-co-glycolide)

D.Lipids, and Liposomes

The desired polynucleotide or polypeptide can also be encapsulated in lipids or packaged in liposomes prior to delivery to the subject or to cells derived therefrom.

Lipid encapsulation is generally accomplished using liposomes which are able to stably bind or entrap and retain nucleic acid. The ratio of condensed polynucleotide or polypeptide to lipid preparation can vary but will generally be around 1:1 (mg DNA:micromoles lipid), or more of lipid. For a review of the use of liposomes as carriers for delivery of nucleic acids, see, Hug and Sleight (1991) *Biochim. Biophys. Acta.* 1097:1-17; Straubinger (1983) *Meth. Enzymol.* 101:512-527.

Liposomal preparations for use in the present invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner (1987) *Proc. Natl. Acad. Sci. USA* 84:7413-7416); mRNA (Malone (1989) *Proc. Natl. Acad. Sci. USA* 86:6077-6081); and purified transcription factors (Debs (1990) *J. Biol. Chem.* 265:10189-10192), in functional form.

Cationic liposomes are readily available. For example, N[1-2,3-dioleoyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. (See, also, Felgner *supra*). Other commercially available liposomes include transfectace (DDAB/DOPE) and

DOTAP/DOPE (Boehringer). Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, eg. Szoka (1978) *Proc. Natl. Acad. Sci. USA* 75:4194-4198; WO90/11092 for a description of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphosphatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

The liposomes can comprise multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs). The various liposome-nucleic acid complexes are prepared using methods known in the art. See eg. Straubinger (1983) *Meth. Immunol.* 101:512-527; Szoka (1978) *Proc. Natl. Acad. Sci. USA* 75:4194-4198; Papahadjopoulos (1975) *Biochim. Biophys. Acta* 394:483; Wilson (1979) *Cell* 17:77; Deamer & Bangham (1976) *Biochim. Biophys. Acta* 443:629; Ostro (1977) *Biochem. Biophys. Res. Commun.* 76:836; Fraley (1979) *Proc. Natl. Acad. Sci. USA* 76:3348; Enoch & Strittmatter (1979) *Proc. Natl. Acad. Sci. USA* 76:145; Fraley (1980) *J. Biol. Chem.* (1980) 255:10431; Szoka & Papahadjopoulos (1978) *Proc. Natl. Acad. Sci. USA* 75:145; and Schaefer-Ridder (1982) *Science* 215:166.

E. Lipoproteins

In addition, lipoproteins can be included with the polynucleotide or polypeptide to be delivered. Examples of lipoproteins to be utilized include: chylomicrons, HDL, IDL, LDL, and VLDL. Mutants, fragments, or fusions of these proteins can also be used. Also, modifications of naturally occurring lipoproteins can be used, such as acetylated LDL. These lipoproteins can target the delivery of polynucleotides to cells expressing lipoprotein receptors. Preferably, if lipoproteins are including with the polynucleotide to be delivered, no other targeting ligand is included in the composition.

Naturally occurring lipoproteins comprise a lipid and a protein portion. The protein portion are known as apoproteins. At the present, apoproteins A, B, C, D, and E have been

isolated and identified. At least two of these contain several proteins, designated by Roman numerals, AI, AII, AIV; CI, CII, CIII.

A lipoprotein can comprise more than one apoprotein. For example, naturally occurring chylomicrons comprises of A, B, C, and E, over time these lipoproteins lose A and acquire C and E apoproteins. VLDL comprises A, B, C, and E apoproteins, LDL comprises apoprotein B; and HDL comprises apoproteins A, C, and E.

The amino acid of these apoproteins are known and are described in, for example, Breslow (1985) *Annu Rev. Biochem* 54:699; Law (1986) *Adv. Exp Med. Biol.* 151:162; Chen (1986) *J Biol Chem* 261:12918; Kane (1980) *Proc Natl Acad Sci USA* 77:2465; and Utermann (1984) *Hum Genet* 65:232.

Lipoproteins contain a variety of lipids including, triglycerides, cholesterol (free and esters), and phospholipids. The composition of the lipids varies in naturally occurring lipoproteins. For example, chylomicrons comprise mainly triglycerides. A more detailed description of the lipid content of naturally occurring lipoproteins can be found, for example, in *Meth. Enzymol.* 128 (1986). The composition of the lipids are chosen to aid in conformation of the apoprotein for receptor binding activity. The composition of lipids can also be chosen to facilitate hydrophobic interaction and association with the polynucleotide binding molecule.

Naturally occurring lipoproteins can be isolated from serum by ultracentrifugation, for instance. Such methods are described in *Meth. Enzymol.* (*supra*); Pitas (1980) *J. Biochem.* 255:5454-5460 and Mahey (1979) *J Clin. Invest* 64:743-750.

Lipoproteins can also be produced by *in vitro* or recombinant methods by expression of the apoprotein genes in a desired host cell. See, for example, Atkinson (1986) *Annu Rev Biophys Chem* 15:403 and Radding (1958) *Biochim Biophys Acta* 30: 443.

Lipoproteins can also be purchased from commercial suppliers, such as Biomedical Technologies, Inc., Stoughton, Massachusetts, USA.

Further description of lipoproteins can be found in Zuckermann et al., PCT. Appln. No. US97/14465.

F. Polycationic Agents

Polycationic agents can be included, with or without lipoprotein, in a composition with the desired polynucleotide or polypeptide to be delivered.

Polycationic agents, typically, exhibit a net positive charge at physiological relevant pH and are capable of neutralizing the electrical charge of nucleic acids to facilitate delivery to a desired location. These agents have both in vitro, ex vivo, and in vivo applications. Polycationic agents can be used to deliver nucleic acids to a living subject either intramuscularly, subcutaneously, etc.

The following are examples of useful polypeptides as polycationic agents: polylysine, polyarginine, polyornithine, and protamine. Other examples include histones, protamines, human serum albumin, DNA binding proteins, non-histone chromosomal proteins, coat proteins from DNA viruses, such as (X174, transcriptional factors also contain domains that bind DNA and therefore may be useful as nucleic acid condensing agents. Briefly, transcriptional factors such as C/CEBP, c-jun, c-fos, AP-1, AP-2, AP-3, CPF, Prot-1, Sp-1, Oct-1, Oct-2, CREP, and TFIID contain basic domains that bind DNA sequences.

Organic polycationic agents include: spermine, spermidine, and putrescine.

The dimensions and of the physical properties of a polycationic agent can be extrapolated from the list above, to construct other polypeptide polycationic agents or to produce synthetic polycationic agents.

Synthetic Polycationic Agents

Synthetic polycationic agents which are useful include, for example, DEAE-dextran, polybrene. Lipofectin[®], and lipofectAMINE[®] are monomers that form polycationic complexes when combined with polynucleotides or polypeptides.

Immunodiagnostic Assays

Neisserial antigens of the invention can be used in immunoassays to detect antibody levels (or, conversely, anti-Neisserial antibodies can be used to detect antigen levels). Immunoassays based on well defined, recombinant antigens can be developed to replace invasive diagnostics methods. Antibodies to Neisserial proteins within biological samples, including for example, blood or serum samples, can be detected. Design of the immunoassays is subject to a great deal of variation, and a variety of these are known in the art. Protocols for the immunoassay may be based, for example, upon competition, or direct reaction, or sandwich type assays. Protocols may also, for example, use solid supports, or may be by immunoprecipitation. Most assays involve the use of labeled antibody or polypeptide; the labels may be, for example, fluorescent, chemiluminescent, radioactive, or dye molecules. Assays which amplify the signals from the probe are also known; examples of which are

assays which utilize biotin and avidin, and enzyme-labeled and mediated immunoassays, such as ELISA assays.

Kits suitable for immunodiagnosis and containing the appropriate labeled reagents are constructed by packaging the appropriate materials, including the compositions of the invention, in suitable containers, along with the remaining reagents and materials (for example, suitable buffers, salt solutions, *etc.*) required for the conduct of the assay, as well as suitable set of assay instructions.

Nucleic Acid Hybridisation

"Hybridization" refers to the association of two nucleic acid sequences to one another by hydrogen bonding. Typically, one sequence will be fixed to a solid support and the other will be free in solution. Then, the two sequences will be placed in contact with one another under conditions that favor hydrogen bonding. Factors that affect this bonding include: the type and volume of solvent; reaction temperature; time of hybridization; agitation; agents to block the non-specific attachment of the liquid phase sequence to the solid support (Denhardt's reagent or BLOTTO); concentration of the sequences; use of compounds to increase the rate of association of sequences (dextran sulfate or polyethylene glycol); and the stringency of the washing conditions following hybridization. See Sambrook *et al.* [*supra*] Volume 2, chapter 9, pages 9.47 to 9.57.

"Stringency" refers to conditions in a hybridization reaction that favor association of very similar sequences over sequences that differ. For example, the combination of temperature and salt concentration should be chosen that is approximately 120 to 200°C below the calculated T_m of the hybrid under study. The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the sequence of interest and then washed under conditions of different stringencies. See Sambrook *et al.* at page 9.50.

Variables to consider when performing, for example, a Southern blot are (1) the complexity of the DNA being blotted and (2) the homology between the probe and the sequences being detected. The total amount of the fragment(s) to be studied can vary a magnitude of 10, from 0.1 to 1 µg for a plasmid or phage digest to 10^{-9} to 10^{-8} g for a single copy gene in a highly complex eukaryotic genome. For lower complexity polynucleotides, substantially shorter blotting, hybridization, and exposure times, a smaller amount of starting polynucleotides, and lower specific activity of probes can be used. For example, a single-copy

yeast gene can be detected with an exposure time of only 1 hour starting with 1 μg of yeast DNA, blotting for two hours, and hybridizing for 4-8 hours with a probe of 10^8 cpm/ μg . For a single-copy mammalian gene a conservative approach would start with 10 μg of DNA, blot overnight, and hybridize overnight in the presence of 10% dextran sulfate using a probe of greater than 10^8 cpm/ μg , resulting in an exposure time of ~24 hours.

Several factors can affect the melting temperature (T_m) of a DNA-DNA hybrid between the probe and the fragment of interest, and consequently, the appropriate conditions for hybridization and washing. In many cases the probe is not 100% homologous to the fragment. Other commonly encountered variables include the length and total G+C content of the hybridizing sequences and the ionic strength and formamide content of the hybridization buffer. The effects of all of these factors can be approximated by a single equation:

$$T_m = 81 + 16.6(\log_{10} C_i) + 0.4\%[(G + C)] - 0.6(\% \text{formamide}) - 600/n - 1.5(\% \text{mismatch}).$$

where C_i is the salt concentration (monovalent ions) and n is the length of the hybrid in base pairs (slightly modified from Meinkoth & Wahl (1984) *Anal. Biochem.* 138: 267-284).

In designing a hybridization experiment, some factors affecting nucleic acid hybridization can be conveniently altered. The temperature of the hybridization and washes and the salt concentration during the washes are the simplest to adjust. As the temperature of the hybridization increases (*ie.* stringency), it becomes less likely for hybridization to occur between strands that are nonhomologous, and as a result, background decreases. If the radiolabeled probe is not completely homologous with the immobilized fragment (as is frequently the case in gene family and interspecies hybridization experiments), the hybridization temperature must be reduced, and background will increase. The temperature of the washes affects the intensity of the hybridizing band and the degree of background in a similar manner. The stringency of the washes is also increased with decreasing salt concentrations.

In general, convenient hybridization temperatures in the presence of 50% formamide are 42°C for a probe with is 95% to 100% homologous to the target fragment, 37°C for 90% to 95% homology, and 32°C for 85% to 90% homology. For lower homologies, formamide content should be lowered and temperature adjusted accordingly, using the equation above. If the homology between the probe and the target fragment are not known, the simplest approach is to start with both hybridization and wash conditions which are nonstringent. If non-specific bands or high background are observed after autoradiography, the filter can be

washed at high stringency and reexposed. If the time required for exposure makes this approach impractical, several hybridization and/or washing stringencies should be tested in parallel.

Nucleic Acid Probe Assays

Methods such as PCR, branched DNA probe assays, or blotting techniques utilizing nucleic acid probes according to the invention can determine the presence of cDNA or mRNA. A probe is said to "hybridize" with a sequence of the invention if it can form a duplex or double stranded complex, which is stable enough to be detected.

The nucleic acid probes will hybridize to the Neisserial nucleotide sequences of the invention (including both sense and antisense strands). Though many different nucleotide sequences will encode the amino acid sequence, the native Neisserial sequence is preferred because it is the actual sequence present in cells. mRNA represents a coding sequence and so a probe should be complementary to the coding sequence; single-stranded cDNA is complementary to mRNA, and so a cDNA probe should be complementary to the non-coding sequence.

The probe sequence need not be identical to the Neisserial sequence (or its complement) — some variation in the sequence and length can lead to increased assay sensitivity if the nucleic acid probe can form a duplex with target nucleotides, which can be detected. Also, the nucleic acid probe can include additional nucleotides to stabilize the formed duplex. Additional Neisserial sequence may also be helpful as a label to detect the formed duplex. For example, a non-complementary nucleotide sequence may be attached to the 5' end of the probe, with the remainder of the probe sequence being complementary to a Neisserial sequence. Alternatively, non-complementary bases or longer sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the a Neisserial sequence in order to hybridize therewith and thereby form a duplex which can be detected.

The exact length and sequence of the probe will depend on the hybridization conditions, such as temperature, salt condition and the like. For example, for diagnostic applications, depending on the complexity of the analyte sequence, the nucleic acid probe typically contains at least 10-20 nucleotides, preferably 15-25, and more preferably at least 30 nucleotides, although it may be shorter than this. Short primers generally require cooler temperatures to form sufficiently stable hybrid complexes with the template.

Probes may be produced by synthetic procedures, such as the triester method of Matteucci *et al.* [*J. Am. Chem. Soc.* (1981) 103:3185], or according to Urdea *et al.* [*Proc. Natl. Acad. Sci. USA* (1983) 80: 7461], or using commercially available automated oligonucleotide synthesizers.

The chemical nature of the probe can be selected according to preference. For certain applications, DNA or RNA are appropriate. For other applications, modifications may be incorporated *eg.* backbone modifications, such as phosphorothioates or methylphosphonates, can be used to increase *in vivo* half-life, alter RNA affinity, increase nuclease resistance *etc.* [*eg.* see Agrawal & Iyer (1995) *Curr Opin Biotechnol* 6:12-19; Agrawal (1996) *TIBTECH* 14:376-387]; analogues such as peptide nucleic acids may also be used [*eg.* see Corey (1997) *TIBTECH* 15:224-229; Buchardt *et al.* (1993) *TIBTECH* 11:384-386].

One example of a nucleotide hybridization assay is described by Urdea *et al.* in international patent application WO92/02526 [see also US patent 5,124,246].

Alternatively, the polymerase chain reaction (PCR) is another well-known means for detecting small amounts of target nucleic acids. The assay is described in: Mullis *et al.* [*Meth. Enzymol.* (1987) 155: 335-350]; US patent 4,683,195; and US patent 4,683,202. Two "primer" nucleotides hybridize with the target nucleic acids and are used to prime the reaction. The primers can comprise sequence that does not hybridize to the sequence of the amplification target (or its complement) to aid with duplex stability or, for example, to incorporate a convenient restriction site. Typically, such sequence will flank the desired Neisserial sequence.

A thermostable polymerase creates copies of target nucleic acids from the primers using the original target nucleic acids as a template. After a threshold amount of target nucleic acids are generated by the polymerase, they can be detected by more traditional methods, such as Southern blots. When using the Southern blot method, the labelled probe will hybridize to the Neisserial sequence (or its complement).

Also, mRNA or cDNA can be detected by traditional blotting techniques described in Sambrook *et al.* [*supra*]. mRNA, or cDNA generated from mRNA using a polymerase enzyme, can be purified and separated using gel electrophoresis. The nucleic acids on the gel are then blotted onto a solid support, such as nitrocellulose. The solid support is exposed to a labelled probe and then washed to remove any unhybridized probe. Next, the duplexes

containing the labeled probe are detected. Typically, the probe is labelled with a radioactive moiety.

EXAMPLES

The examples describe nucleic acid sequences which have been identified in *N. meningitidis*, and *N. gonorrhoeae* along with their respective and putative translation products. Not all of the nucleic acid sequences are complete *ie.* they encode less than the full-length wild-type protein.

The examples are generally in the following format:

- a nucleotide sequence which has been identified in *N. meningitidis*
- the putative translation product of said *N. meningitidis* sequence
- a computer analysis of said translation product based on database comparisons
- a corresponding nucleotide sequence identified from *N. gonorrhoeae*
- the putative translation product of said *N. gonorrhoeae* sequence
- a comparison of the percentage of identity between the translation product of the *N. meningitidis* sequence and the *N. gonorrhoeae* sequence.
- a corresponding nucleotide sequence identified from strain A of *N. meningitidis*
- the putative translation product of said *N. meningitidis* strain A sequence
- a comparison of the percentage of identity between the translation product of the *N. meningitidis* sequence and the *N. gonorrhoeae* sequence.
- a description of the characteristics of the protein which indicates that it might be suitably antigenic or immunogenic.

Sequence comparisons were performed at NCBI (<http://www.ncbi.nlm.nih.gov>) using the algorithms BLAST, BLAST2, BLASTn, BLASTp, tBLASTn, BLASTx, & tBLASTx [eg. see also Altschul *et al.* (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25:2289-3402]. Searches were performed against the following databases: non-redundant GenBank+EMBL+DDBJ+PDB sequences and non-redundant GenBank CDS translations+PDB+SwissProt+SPupdate+PIR sequences.

Dots within nucleotide sequences represent nucleotides which have been arbitrarily introduced in order to maintain a reading frame. In the same way, double-underlined nucleotides were removed. Lower case letters represent ambiguities which arose during

alignment of independent sequencing reactions (some of the nucleotide sequences in the examples are derived from combining the results of two or more experiments).

Nucleotide sequences were scanned in all six reading frames to predict the presence of hydrophobic domains using an algorithm based on the statistical studies of Esposti *et al.* [Critical evaluation of the hydropathy of membrane proteins (1990) *Eur J Biochem* 190:207-219]. These domains represent potential transmembrane regions or hydrophobic leader sequences.

Open reading frames were predicted from fragmented nucleotide sequences using the program ORFFINDER (NCBI).

Underlined amino acid sequences indicate possible transmembrane domains or leader sequences in the ORFs, as predicted by the PSORT algorithm (<http://www.psort.nibb.ac.jp>). Functional domains were also predicted using the MOTIFS program (GCG Wisconsin & PROSITE).

For each of the following examples: based on the presence of a putative leader sequence and/or several putative transmembrane domains (single-underlined) in the gonococcal protein, it is predicted that the proteins from *N. meningitidis* and *N. gonorrhoeae*, and their respective epitopes, could be useful antigens or immunogenic compositions for vaccines or diagnostics.

The standard techniques and procedures which may be employed in order to perform the invention (*e.g.* to utilize the disclosed sequences for vaccination or diagnostic purposes) were summarized above. This summary is not a limitation on the invention but, rather, gives examples that may be used, but are not required.

In particular, the following methods were used to express, purify and biochemically characterize the proteins of the invention.

Chromosomal DNA Preparation

N.meningitidis strain 2996 was grown to exponential phase in 100ml of GC medium, harvested by centrifugation, and resuspended in 5ml buffer (20%(w/v) Sucrose, 50mM Tris-HCl, 50mM EDTA, pH8). After 10 minutes incubation on ice, the bacteria were lysed by adding 10ml of lysis solution (50mM NaCl, 1% Na-Sarkosyl, 50µg/ml Proteinase K), and the suspension incubated at 37°C for 2 hours. Two phenol extractions (equilibrated to pH 8) and one CHCl₃/isoamylalcohol (24:1) extraction were performed. DNA was precipitated by addition of 0.3M sodium acetate and 2 volumes of ethanol, and collected by centrifugation.

The pellet was washed once with 70%(v/v) ethanol and redissolved in 4.0ml TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). The DNA concentration was measured by reading the OD at 260 nm.

Oligonucleotide design

Synthetic oligonucleotide primers were designed on the basis of the coding sequence of each ORF, using (a) the meningococcus B sequence when available, or (b) the gonococcus/meningococcus A sequence, adapted to the codon preference usage of meningococcus as necessary. Any predicted signal peptides were omitted, by designing the 5' primers to sequence immediately downstream from the predicted leader sequence.

For most ORFs, the 5' primers included two restriction enzyme recognition sites (*Bam*HI-*Nde*I, *Bam*HI-*Nhe*I, *Eco*RI-*Nde*I or *Eco*RI-*Nhe*I), depending on the restriction pattern of the gene of interest. The 3' primers included a *Xho*I or a *Hind*III restriction site (table 1). This procedure was established in order to direct the cloning of each amplification product (corresponding to each ORF) into two different expression systems: pGEX-KG (using *Bam*HI-*Xho*I, *Bam*HI-*Hind*III, *Eco*RI-*Xho*I or *Eco*RI-*Hind*III), and pET21b+ (using *Nde*I-*Xho*I, *Nhe*I-*Xho*I, *Nde*I-*Hind*III or *Nhe*I-*Hind*III).

5'-end primer tail:	<u>CGCGGATCCCATATG</u>	(<i>Bam</i> HI- <i>Nde</i> I)
	<u>CGCGGATCCGCTAGC</u>	(<i>Bam</i> HI- <i>Nhe</i> I)
	<u>CCGGAATTCTACATATG</u>	(<i>Eco</i> RI- <i>Nde</i> I)
	<u>CCGGAATTCTAGCTAGC</u>	(<i>Eco</i> RI- <i>Nhe</i> I)
3'-end primer tail:	<u>CCCGCTCGAG</u>	(<i>Xho</i> I)
	<u>CCCGCTCGAG</u>	(<i>Hind</i> III)

For cloning ORFs into the pGEX-His vector, the 5' and 3' primers contained only one restriction enzyme site (*Eco*RI, *Kpn*I or *Sal*I for the 5' primers and *Pst*I, *Xba*I, *Sph*I or *Sal*I for the 3' primers). Again restriction sites were chosen according to the particular restriction pattern of the gene (table 1).

5'-end primer tail:	(AAA) <u>AAAGAATTC</u>	(<i>Eco</i> RI)
	(AAA) <u>AAAGGTACC</u>	(<i>Kpn</i> I)
3'-end primer tail:	(AAA) <u>AAACTGCAG</u>	(<i>Pst</i> I)
	(AAA) <u>AAATCTAGA</u>	(<i>Xba</i> I)

$$T_m = 4 (G+C) + 2 (A+T) \quad (\text{tail excluded})$$

$$T_m = 64.9 + 0.41 (\% \text{ GC}) - 600/N \quad (\text{whole primer})$$

Oligonucleotides were synthesized using a Perkin Elmer 394 DNA/RNA Synthesizer, eluted from the columns in 2.0ml NH_4OH , and deprotected by 5 hours incubation at 56°C . The oligos were precipitated by addition of 0.3M Na-Acetate and 2 volumes ethanol. The samples were centrifuged and the pellets resuspended in either 100 μl or 1.0ml of water. The OD_{260} was determined using a Perkin Elmer Lambda Bio spectrophotometer and the concentration adjusted to 2-10pmol/ μl .

The standard PCR protocol was as follows: 50-200ng of genomic DNA was used as a template in the presence of 20-40μM of each oligonucleotide primer, 400-800μM dNTPs solution, 1x PCR buffer (including 1.5mM MgCl₂), 2.5 units *TaqI* DNA polymerase (using

Perkin-Elmer AmpliTaq, GIBCO Platinum, Pwo DNA polymerase, or Tahara Shuzo Taq polymerase). In some cases, PCR was optimised by the addition of 10µl DMSO or 50µl 2M Betaine.

After a hot start (adding the polymerase during a preliminary 3 minute incubation of the whole mix at 95°C), each sample underwent a two-step amplification. The first 5 cycles were performed using the hybridization temperature that excluded the restriction enzyme tail of the primer (see above). This was followed by 30 cycles using the hybridization temperature calculated for the whole length oligos. The cycles were completed with a 10 minute extension step at 72°C. The standard cycles were as follows:

	Denaturation	Hybridisation	Elongation
First 5 cycles	30 seconds 95°C	30 seconds 50-55°C	30-60 seconds 72°C
Last 30 cycles	30 seconds 95°C	30 seconds 65-70°C	30-60 seconds 72°C

Elongation times varied according to the length of the ORF to be amplified. Amplifications were performed using either a 9600 or a 2400 Perkin Elmer GeneAmp PCR System. To check the results, 1/10 of the amplification volume was loaded onto a 1-1.5% (w/v) agarose gel and the size of each amplified fragment compared with a DNA molecular weight marker.

The amplified DNA was either loaded directly on a 1% agarose gel or first precipitated with ethanol and resuspended in a volume suitable to be loaded on a 1.0% agarose gel. The DNA fragment corresponding to the band of correct size was purified using the Qiagen Gel Extraction Kit, following the manufacturer's protocol. DNA fragments were eluted in a volume of 30µl or 50µl with either H₂O or 10mM Tris, pH 8.5.

Digestion of PCR fragments

The purified DNA corresponding to the amplified fragment was doubly-digested with the appropriate restriction enzymes for; cloning into pET-21b+ and expressing the protein as a C-terminus His-tagged fusion, for cloning into pGEX-KG and expressing the protein as a N-

terminus GST-fusion, and for cloning into pGEX-His and expressing the protein as a N-terminus GST-His tagged fusion.

Each purified DNA fragment was incubated at 37°C for 3 hours to overnight with 20 units of appropriate restriction enzyme (New England Biolabs) in a volume of either 30 or 40 µl in the presence of suitable digestion buffer. Digested fragments were purified using the QIAquick PCR purification kit (following the manufacturer's instructions) and eluted in a volume of 30 µl or 50 µl with either H₂O or 10mM Tris, pH 8.5. The DNA concentration was determined by quantitative agarose gel electrophoresis (1.0% gel) in the presence of a titrated molecular weight marker.

Digestion of the cloning vectors (pET22B, pGEX-KG, pTRC-His A, pET21b+, pGEX-KG, and pGEX-His)

The vector pGEX-His is a modified pGEX-2T vector carrying a region encoding six histidine residues upstream of the thrombin cleavage site and containing the multiple cloning site of the vector pTRC99 (Pharmacia). 10 µg plasmid was double-digested with 50 units of each restriction enzyme in 200 µl reaction volume in the presence of appropriate buffer by overnight incubation at 37°C. After loading the whole digestion on a 1% agarose gel, the band corresponding to the digested vector was purified from the gel using the Qiagen QIAquick Gel Extraction Kit and the DNA was eluted in 50 µl of 10 mM Tris-HCl, pH 8.5. The DNA concentration was evaluated by measuring OD₂₆₀ of the sample, and adjusted to 50 µg/µl. 1 µl of plasmid was used for each cloning procedure.

10 µg of plasmid vector was doubly-digested with 50 units of each restriction enzyme in a volume of 200 µl with the appropriate buffer overnight at 37°C. The digest was loaded onto a 1.0% agarose gel and the band corresponding to the digested vector purified using the Qiagen QIAquick Gel Extraction Kit. DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5. The DNA concentration was evaluated by measuring OD_{260nm} and the concentration adjusted to 50 µg/µl. 1 µl of plasmid was used for each cloning procedure.

Cloning

For some ORFs, the fragments corresponding to each ORF, previously digested and purified, were ligated in both pET22b and pGEX-KG. In a final volume of 20 µl, a molar

ratio of 3:1 fragment/vector was ligated using 0.5 μ l of NEB T4 DNA ligase (400 units/ μ l), in the presence of the buffer supplied by the manufacturer. The reaction was incubated at room temperature for 3 hours. In some experiments, ligation was performed using the Boehringer "Rapid Ligation Kit", following the manufacturer's instructions.

In order to introduce the recombinant plasmid in a suitable strain, 100 μ l *E. coli* DH5 competent cells were incubated with the ligase reaction solution for 40 minutes on ice, then at 37°C for 3 minutes, then, after adding 800 μ l LB broth, again at 37°C for 20 minutes. The cells were then centrifuged at maximum speed in an Eppendorf microfuge and resuspended in approximately 200 μ l of the supernatant. The suspension was then plated on LB ampicillin (100 mg/ml).

The screening of the recombinant clones was performed by growing 5 randomly-chosen colonies overnight at 37 °C in either 2 ml (pGEX or pTC clones) or 5ml (pET clones) LB broth + 100 μ g/ml ampicillin. The cells were then pelleted and the DNA extracted using the Qiagen QIAprep Spin Miniprep Kit, following the manufacturer's instructions, to a final volume of 30 μ l. 5 μ l of each individual miniprep (approximately 1g) were digested with either *NdeI/XhoI* or *BamHI/XhoI* and the whole digestion loaded onto a 1-1.5% agarose gel (depending on the expected insert size), in parallel with the molecular weight marker (1Kb DNA Ladder, GIBCO). The screening of the positive clones was made on the base of the correct insert size.

For other ORFs, the fragments corresponding to each ORF, previously digested and purified, were ligated into both pET21b+ and pGEX-KG. A molar ratio of of 3:1 fragment/vector was used in a final volume of 20 μ l, that included 0.5 μ l T4 DNA ligase (400 units/ μ l, NEB) and ligation buffer supplied by the manufacturer. The reaction was performed at room temperature for 3 hours. In some experiments, ligation was performed using the Boehringer "Rapid Ligation Kit" and the manufacturer's protocol.

Recombinant plasmid was transformed into 100 μ l of competent *E. coli* DH5 or HB101 by incubating the ligase reaction solution and bacteria for 40 minutes on ice then at 37°C for 3 minutes. This was followed by the addition of 800 μ l LB broth and incubation at 37°C for 20 minutes. The cells were centrifuged at maximum speed in an Eppendorf microfuge, resuspended in approximately 200 μ l of the supernatant and plated onto LB ampicillin (100mg/ml) agar.

Screening for recombinant clones was performed by growing 5 randomly selected colonies overnight at 37°C in either 2.0ml (pGEX-KG clones) or 5.0ml (pET clones) LB broth + 100µg/ml ampicillin. Cells were pelleted and plasmid DNA extracted using the Qiagen QIAprep Spin Miniprep Kit, following the manufacturer's instructions. Approximately 1µg of each individual miniprep was digested with the appropriate restriction enzymes and the digest loaded onto a 1-1.5% agarose gel (depending on the expected insert size), in parallel with the molecular weight marker (1kb DNA Ladder, GIBCO). Positive clones were selected on the basis of the size of insert.

ORFs were cloned into PGEX-His, by doubly-digesting the PCR product and ligating into similarly digested vector. After cloning, recombinant plasmids were transformed into the *E.coli* host W3110. Individual clones were grown overnight at 37°C in LB broth with 50µg/ml ampicillin.

Certain ORFs may be cloned into the pGEX-HIS vector using *EcoRI-PstI* cloning sites, or *EcoRI-SalI*, or *SalI-PstI*. After cloning, the recombinant plasmids may be introduced in the *E.coli* host W3110.

Expression

Each ORF cloned into the expression vector may then be transformed into the strain suitable for expression of the recombinant protein product. 1 µl of each construct was used to transform 30 µl of *E.coli* BL21 (pGEX vector), *E.coli* TOP 10 (pTRC vector) or *E.coli* BL21-DE3 (pET vector), as described above. In the case of the pGEX-His vector, the same *E.coli* strain (W3110) was used for initial cloning and expression. Single recombinant colonies were inoculated into 2ml LB+Amp (100 µg/ml), incubated at 37°C overnight, then diluted 1:30 in 20 ml of LB+Amp (100 µg/ml) in 100 ml flasks, making sure that the OD₆₀₀ ranged between 0.1 and 0.15. The flasks were incubated at 30°C into gyratory water bath shakers until OD indicated exponential growth suitable for induction of expression (0.4-0.8 OD for pET and pTRC vectors; 0.8-1 OD for pGEX and pGEX-His vectors). For the pET, pTRC and pGEX-His vectors, the protein expression was induced by addition of 1mM IPTG, whereas in the case of pGEX system the final concentration of IPTG was 0.2 mM. After 3 hours incubation at 30°C, the final concentration of the sample was checked by OD. In order to check expression, 1ml of each sample was removed, centrifuged in a microfuge, the pellet

resuspended in PBS, and analysed by 12% SDS-PAGE with Coomassie Blue staining. The whole sample was centrifuged at 6000g and the pellet resuspended in PBS for further use.

GST-fusion proteins large-scale purification.

For some ORFs, a single colony was grown overnight at 37°C on LB+Amp agar plate. The bacteria were inoculated into 20 ml of LB+Amp liquid culture in a water bath shaker and grown overnight. Bacteria were diluted 1:30 into 600 ml of fresh medium and allowed to grow at the optimal temperature (20-37°C) to OD₅₅₀ 0.8-1. Protein expression was induced with 0.2mM IPTG followed by three hours incubation. The culture was centrifuged at 8000 rpm at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 7.5 ml cold PBS. The cells were disrupted by sonication on ice for 30 sec at 40W using a Branson sonifier B-15, frozen and thawed two times and centrifuged again. The supernatant was collected and mixed with 150µl Glutathione-Sepharose 4B resin (Pharmacia) (previously washed with PBS) and incubated at room temperature for 30 minutes. The sample was centrifuged at 700g for 5 minutes at 4°C. The resin was washed twice with 10 ml cold PBS for 10 minutes, resuspended in 1ml cold PBS, and loaded on a disposable column. The resin was washed twice with 2ml cold PBS until the flow-through reached OD₂₈₀ of 0.02-0.06. The GST-fusion protein was eluted by addition of 700µl cold Glutathione elution buffer (10mM reduced glutathione, 50mM Tris-HCl) and fractions collected until the OD₂₈₀ was 0.1. 21µl of each fraction were loaded on a 12% SDS gel using either Biorad SDS-PAGE Molecular weight standard broad range (M1) (200, 116.25, 97.4, 66.2, 45, 31, 21.5, 14.4, 6.5 kDa) or Amersham Rainbow Marker (M") (220, 66, 46, 30, 21.5, 14.3 kDa) as standards. As the MW of GST is 26kDa, this value must be added to the MW of each GST-fusion protein.

For other ORFs, for each clone to be purified as a GST-fusion, a single colony was streaked out and grown overnight at 37°C on a LB/Amp. (100µg/ml) agar plate. An isolated colony from this plate was inoculated into 20ml of LB/Amp (100 µg/ml) liquid medium and grown overnight at 37°C with shaking. The overnight culture was diluted 1:30 into 600ml LB/Amp (100µg/ml) liquid medium and allowed to grow at the optimal temperature (20-37°C) until the OD_{550nm} reached 0.6-0.8. Recombinant protein expression was induced by addition of IPTG (final concentration 0.2mM) and the culture incubated for a further 3 hours. Bacteria were harvested by centrifugation at 8000xg for 15 min at 4°C.

The bacterial pellet was resuspended in 7.5ml cold PBS. Cells were disrupted by sonication on ice four times for 30 sec at 40W using a Branson sonifier 450 and centrifuged at 13 000xg for 30 min at 4°C. The supernatant was collected and mixed with 150µl Glutathione-Sepharose 4B resin (Pharmacia), previously equilibrated with PBS, and incubated at room temperature with gentle agitation for 30 min. The batch-wise preparation was centrifuged at 700xg for 5 min at 4°C and the supernatant discarded. The resin was washed twice (batchwise) with 10ml cold PBS for 10 min, resuspended in 1ml cold PBS, and loaded onto a disposable column. The resin continued to be washed with cold PBS, until the OD_{280nm} of the flow-through reached 0.02-0.01. The GST-fusion protein was eluted by addition of 700µl cold glutathione elution buffer (10mM reduced glutathione, 50mM Tris-HCl pH 8.0) and fractions collected, until the OD_{280nm} of the eluate indicated all the recombinant protein was obtained. 20µl aliquots of each elution fraction were analyzed by SDS-PAGE using a 12% gel. The molecular mass of the purified proteins was determined using either the Bio-Rad broad range molecular weight standard (M1) (200, 116, 97.4, 66.2, 45.0, 31.0, 21.5, 14.4, 6.5 kDa) or the Amersham Rainbow Marker (M2) (220, 66.2, 46.0, 30.0, 21.5, 14.3 kDa). The molecular weights of GST-fusion proteins are a combination of the 26 kDa GST protein and its fusion partner. Protein concentrations were estimated using the Bradford assay.

His-fusion soluble proteins large-scale purification.

For some ORFs, a single colony was grown overnight at 37°C on a LB + Amp agar plate. The bacteria were inoculated into 20ml of LB+Amp liquid culture and incubated overnight in a water bath shaker. Bacteria were diluted 1:30 into 600ml fresh medium and allowed to grow at the optimal temperature (20-37°C) to OD₅₅₀ 0.6-0.8. Protein expression was induced by addition of 1 mM IPTG and the culture further incubated for three hours. The culture was centrifuged at 8000 rpm at 4°C, the supernatant was discarded and the bacterial pellet was resuspended in 7.5ml cold 10mM imidazole buffer (300 mM NaCl, 50 mM phosphate buffer, 10 mM imidazole, pH 8). The cells were disrupted by sonication on ice for 30 sec at 40W using a Branson sonifier B-15, frozen and thawed two times and centrifuged again. The supernatant was collected and mixed with 150µl Ni²⁺-resin (Pharmacia) (previously washed with 10mM imidazole buffer) and incubated at room temperature with gentle agitation for 30 minutes. The sample was centrifuged at 700g for 5 minutes at 4°C. The resin was washed twice with 10 ml cold 10mM imidazole buffer for 10 minutes,

resuspended in 1ml cold 10mM imidazole buffer and loaded on a disposable column. The resin was washed at 4°C with 2ml cold 10mM imidazole buffer until the flow-through reached the O.D₂₈₀ of 0.02-0.06. The resin was washed with 2ml cold 20mM imidazole buffer (300 mM NaCl, 50 mM phosphate buffer, 20 mM imidazole, pH 8) until the flow-through reached the O.D₂₈₀ of 0.02-0.06. The His-fusion protein was eluted by addition of 700µl cold 250mM imidazole buffer (300 mM NaCl, 50 mM phosphate buffer, 250 mM imidazole, pH 8) and fractions collected until the O.D₂₈₀ was 0.1. 21µl of each fraction were loaded on a 12% SDS gel.

His-fusion insoluble proteins large-scale purification.

A single colony was grown overnight at 37 °C on a LB + Amp agar plate. The bacteria were inoculated into 20 ml of LB+Amp liquid culture in a water bath shaker and grown overnight. Bacteria were diluted 1:30 into 600ml fresh medium and let to grow at the optimal temperature (37°C) to O.D₅₅₀ 0.6-0.8. Protein expression was induced by addition of 1 mM IPTG and the culture further incubated for three hours. The culture was centrifuged at 8000rpm at 4°C. The supernatant was discarded and the bacterial pellet was resuspended in 7.5 ml buffer B (urea 8M, 10mM Tris-HCl, 100mM phosphate buffer, pH 8.8). The cells were disrupted by sonication on ice for 30 sec at 40W using a Branson sonifier B-15, frozen and thawed twice and centrifuged again. The supernatant was stored at -20°C, while the pellets were resuspended in 2 ml guanidine buffer (6M guanidine hydrochloride, 100mM phosphate buffer, 10 mM Tris-HCl, pH 7.5) and treated in a homogenizer for 10 cycles. The product was centrifuged at 13000 rpm for 40 minutes. The supernatant was mixed with 150µl Ni²⁺-resin (Pharmacia) (previously washed with buffer B) and incubated at room temperature with gentle agitation for 30 minutes. The sample was centrifuged at 700 g for 5 minutes at 4°C. The resin was washed twice with 10 ml buffer B for 10 minutes, resuspended in 1ml buffer B, and loaded on a disposable column. The resin was washed at room temperature with 2ml buffer B until the flow-through reached the OD₂₈₀ of 0.02-0.06. The resin was washed with 2ml buffer C (urea 8M, 10mM Tris-HCl, 100mM phosphate buffer, pH 6.3) until the flow-through reached the O.D₂₈₀ of 0.02-0.06. The His-fusion protein was eluted by addition of 700µl elution buffer (urea 8M, 10mM Tris-HCl, 100mM phosphate buffer, pH 4.5) and fractions collected until the OD₂₈₀ was 0.1. 21µl of each fraction were loaded on a 12% SDS gel.

Purification of His-fusion proteins.

For each clone to be purified as a His-fusion, a single colony was streaked out and grown overnight at 37°C on a LB/Amp (100 µg/ml) agar plate. An isolated colony from this plate was inoculated into 20ml of LB/Amp (100 µg/ml) liquid medium and grown overnight at 37°C with shaking. The overnight culture was diluted 1:30 into 600ml LB/Amp (100 µg/ml) liquid medium and allowed to grow at the optimal temperature (20-37°C) until the OD_{550nm} reached 0.6-0.8. Expression of recombinant protein was induced by addition of IPTG (final concentration 1.0mM) and the culture incubated for a further 3 hours. Bacteria were harvested by centrifugation at 8000xg for 15 min at 4°C.

The bacterial pellet was resuspended in 7.5ml of either (i) cold buffer A (300mM NaCl, 50mM phosphate buffer, 10mM imidazole, pH 8.0) for soluble proteins or (ii) buffer B (8M urea, 10mM Tris-HCl, 100mM phosphate buffer, pH 8.8) for insoluble proteins. Cells were disrupted by sonication on ice four times for 30 sec at 40W using a Branson sonifier 450 and centrifuged at 13 000xg for 30 min at 4°C. For insoluble proteins, pellets were resuspended in 2.0 ml buffer C (6M guanidine hydrochloride, 100mM phosphate buffer, 10mM Tris-HCl, pH 7.5) and treated with a Dounce homogenizer for 10 cycles. The homogenate was centrifuged at 13 000xg for 40 min and the supernatant retained.

Supernatants for both soluble and insoluble preparations were mixed with 150µl Ni²⁺-resin (previously equilibrated with either buffer A or buffer B, as appropriate) and incubated at room temperature with gentle agitation for 30 min. The resin was Chelating Sepharose Fast Flow (Pharmacia), prepared according to manufacturers protocol. The batch-wise preparation was centrifuged at 700xg for 5 min at 4°C and the supernatant discarded. The resin was washed twice (batch-wise) with 10ml buffer A or B for 10 min, resuspended in 1.0 ml buffer A or B and loaded onto a disposable column. The resin continued to be washed with either (i) buffer A at 4°C or (ii) buffer B at room temperature, until the OD_{280nm} of the flow-through reached 0.02-0.01. The resin was further washed with either (i) cold buffer C (300mM NaCl, 50mM phosphate buffer, 20mM imidazole, pH 8.0) or (ii) buffer D (8M urea, 10mM Tris-HCl, 100mM phosphate buffer, pH 6.3) until the the OD_{280nm} of the flow-through reached 0.02-0.01. The His-fusion protein was eluted by addition of 700µl of either (i) cold elution buffer A (300mM NaCl, 50mM phosphate buffer, 250mM imidazole, pH 8.0) or (ii) elution buffer B (8 M urea, 10mM Tris-HCl, 100mM phosphate buffer, pH 4.5) and fractions

collected until the $O.D_{280nm}$ indicated all the recombinant protein was obtained. 20 μ l aliquots of each elution fraction were analyzed by SDS-PAGE using a 12% gel. Protein concentrations were estimated using the Bradford assay.

His-fusion proteins renaturation

In the cases where denaturation was required to solubilize proteins, a renaturation step was employed prior to immunization. Glycerol was added to the denatured fractions obtained above to give a final concentration of 10%(v/v). The proteins were diluted to 200 μ g/ml using dialysis buffer I (10% (v/v) glycerol, 0.5M arginine, 50mM phosphate buffer, 5.0mM reduced glutathione, 0.5mM oxidised glutathione, 2.0M urea, pH 8.8) and dialysed against the same buffer for 12-14 hours at 4°C. Further dialysis was performed with buffer II (10% (v/v) glycerol, 0.5M arginine, 50mM phosphate buffer, 5.0mM reduced glutathione, 0.5mM oxidised glutathione, pH 8.8) for 12-14 hours at 4°C.

Alternatively, 10% glycerol was added to the denatured proteins. The proteins were then diluted to 20 μ g/ml using dialysis buffer I (10% glycerol, 0.5M arginine, 50mM phosphate buffer, 5mM reduced glutathione, 0.5mM oxidised glutathione, 2M urea, pH 8.8) and dialysed against the same buffer at 4°C for 12-14 hours. The protein was further dialysed against dialysis buffer II (10% glycerol, 0.5M arginine, 50mM phosphate buffer, 5mM reduced glutathione, 0.5mM oxidised glutathione, pH 8.8) for 12-14 hours at 4°C.

Protein concentration was evaluated using the formula:

$$\text{Protein (mg/ml)} = (1.55 \times OD_{280}) - (0.76 \times OD_{260})$$

Purification of proteins

To analyse the solubility, pellets obtained from 3.0ml cultures were resuspended in 500 μ l buffer M1 (PBS pH 7.2). 25 μ l of lysozyme (10mg/ml) was added and the bacteria incubated for 15 min at 4°C. Cells were disrupted by sonication on ice four times for 30 sec at 40W using a Branson sonifier 450 and centrifuged at 13 000xg for 30 min at 4°C. The supernatant was collected and the pellet resuspended in buffer M2 [8M urea, 0.5M NaCl, 20mM imidazole and 0.1M NaH_2PO_4] and incubated for 3 to 4 hours at 4°C. After centrifugation, the supernatant was collected and the pellet resuspended in buffer M3 [6M guanidinium-HCl, 0.5M NaCl, 20mM imidazole and 0.1M NaH_2PO_4] overnight at 4°C. The

supernatants from all steps were analysed by SDS-PAGE. Some proteins were found to be soluble in PBS, others need urea or guanidium-HCl for solubilization.

For preparative scale purifications, 500ml cultures were induced and fusion proteins solubilized in either buffer M1, M2 or M3 using the procedure described above. Crude extracts were loaded onto a Ni-NTA superflow column (Quiagen) equilibrated with buffer M1, M2 or M3 depending on the solubilization buffer employed. Unbound material was eluted by washing the column with the same buffer. The recombinant fusion protein was eluted with the corresponding buffer containing 500mM imidazole then dialysed against the same buffer in the absence of imidazole.

Mice immunisations

20µg of each purified protein are used to immunise mice intraperitoneally. In the case of some ORFs, Balb-C mice were immunised with Al(OH)₃ as adjuvant on days 1, 21 and 42, and immune response was monitored in samples taken on day 56. For other ORFs, CD1 mice could be immunised using the same protocol. For ORFs 25 and 40, CD1 mice were immunised using Freund's adjuvant, and the same immunisation protocol was used, except that the immune response was measured on day 42, rather than 56. Similarly, for still other ORFs, CD1 mice were immunised with Freund's adjuvant, but the immune response was measured on day 49. Alternatively, 20µg of each purified protein was mixed with Freund's adjuvant and used to immunise CD1 mice intraperitoneally. For many of the proteins, the immunization was performed on days 1, 21 and 35, and immune response was monitored in samples taken on days 34 and 49. For some proteins, the third immunization was performed on day 28, rather than 35, and the immune response was measured on days 20 and 42, rather than 34 and 49.

ELISA assay (sera analysis)

The acapsulated MenB M7 strain was plated on chocolate agar plates and incubated overnight at 37°C. Bacterial colonies were collected from the agar plates using a sterile dracon swab and inoculated into 7ml of Mueller-Hinton Broth (Difco) containing 0.25% Glucose. Bacterial growth was monitored every 30 minutes by following OD₆₂₀. The bacteria were let to grow until the OD reached the value of 0.3-0.4. The culture was centrifuged for 10 minutes at 10000 rpm. The supernatant was discarded and bacteria were washed once with PBS, resuspended in PBS containing 0.025% formaldehyde, and incubated

for 2 hours at room temperature and then overnight at 4°C with stirring. 100µl bacterial cells were added to each well of a 96 well Greiner plate and incubated overnight at 4°C. The wells were then washed three times with PBT washing buffer (0.1% Tween-20 in PBS). 200 µl of saturation buffer (2.7% Polyvinylpyrrolidone 10 in water) was added to each well and the plates incubated for 2 hours at 37°C. Wells were washed three times with PBT. 200 µl of diluted sera (Dilution buffer: 1% BSA, 0.1% Tween-20, 0.1% NaN₃ in PBS) were added to each well and the plates incubated for 90 minutes at 37°C. Wells were washed three times with PBT. 100 µl of HRP-conjugated rabbit anti-mouse (Dako) serum diluted 1:2000 in dilution buffer were added to each well and the plates were incubated for 90 minutes at 37°C. Wells were washed three times with PBT buffer. 100 µl of substrate buffer for HRP (25 ml of citrate buffer pH5, 10 mg of O-phenildiamine and 10 µl of H₂O) were added to each well and the plates were left at room temperature for 20 minutes. 100 µl H₂SO₄ was added to each well and OD₄₉₀ was followed. The ELISA was considered positive when OD₄₉₀ was 2.5 times the respective pre-immune sera.

Alternatively, The acapsulated MenB M7 strain was plated on chocolate agar plates and incubated overnight at 37°C. Bacterial colonies were collected from the agar plates using a sterile dracon swab and inoculated into Mueller-Hinton Broth (Difco) containing 0.25% Glucose. Bacterial growth was monitored every 30 minutes by following OD₆₂₀. The bacteria were let to grow until the OD reached the value of 0.3-0.4. The culture was centrifuged for 10 minutes at 10 000rpm. The supernatant was discarded and bacteria were washed once with PBS, resuspended in PBS containing 0.025% formaldehyde, and incubated for 1 hour at 37°C and then overnight at 4°C with stirring. 100µl bacterial cells were added to each well of a 96 well Greiner plate and incubated overnight at 4°C. The wells were then washed three times with PBT washing buffer (0.1% Tween-20 in PBS). 200µl of saturation buffer (2.7% Polyvinylpyrrolidone 10 in water) was added to each well and the plates incubated for 2 hours at 37°C. Wells were washed three times with PBT. 200µl of diluted sera (Dilution buffer: 1% BSA, 0.1% Tween-20, 0.1% NaN₃ in PBS) were added to each well and the plates incubated for 2 hours at 37°C. Wells were washed three times with PBT. 100µl of HRP-conjugated rabbit anti-mouse (Dako) serum diluted 1:2000 in dilution buffer were added to each well and the plates were incubated for 90 minutes at 37°C. Wells were washed three times with PBT buffer. 100µl of substrate buffer for HRP (25ml of citrate buffer pH5, 10mg of O-

phenildiamine and 10 μ l of H₂O₂) were added to each well and the plates were left at room temperature for 20 minutes. 100 μ l of 12.5% H₂SO₄ was added to each well and OD₄₉₀ was followed. The ELISA titers were calculated arbitrarily as the dilution of sera which gave an OD₄₉₀ value of 0.4 above the level of preimmune sera. The ELISA was considered positive when the dilution of sera with OD₄₉₀ of 0.4 was higher than 1:400.

FACScan bacteria Binding Assay procedure.

The acapsulated MenB M7 strain was plated on chocolate agar plates and incubated overnight at 37°C. Bacterial colonies were collected from the agar plates using a sterile dracon swab and inoculated into 4 tubes containing 8ml each Mueller-Hinton Broth (Difco) containing 0.25% glucose. Bacterial growth was monitored every 30 minutes by following OD₆₂₀. The bacteria were let to grow until the OD reached the value of 0.35-0.5. The culture was centrifuged for 10 minutes at 4000rpm. The supernatant was discarded and the pellet was resuspended in blocking buffer (1% BSA in PBS, 0.4% NaN₃) and centrifuged for 5 minutes at 4000rpm. Cells were resuspended in blocking buffer to reach OD₆₂₀ of 0.07. 100 μ l bacterial cells were added to each well of a Costar 96 well plate. 100 μ l of diluted (1:100, 1:200, 1:400) sera (in blocking buffer) were added to each well and plates incubated for 2 hours at 4°C. Cells were centrifuged for 5 minutes at 4000rpm, the supernatant aspirated and cells washed by addition of 200 μ l/well of blocking buffer in each well. 100 μ l of R-Phicoerytrin conjugated F(ab)₂ goat anti-mouse, diluted 1:100, was added to each well and plates incubated for 1 hour at 4°C. Cells were spun down by centrifugation at 4000rpm for 5 minutes and washed by addition of 200 μ l/well of blocking buffer. The supernatant was aspirated and cells resuspended in 200 μ l/well of PBS, 0.25% formaldehyde. Samples were transferred to FACScan tubes and read. The condition for FACScan (Laser Power 15mW) setting were: FL2 on; FSC-H threshold:92; FSC PMT Voltage: E 01; SSC PMT: 474; Amp. Gains 6.1; FL-2 PMT: 586; compensation values: 0.

OMV preparations

Bacteria were grown overnight on 5 GC plates, harvested with a loop and resuspended in 10 ml 20mM Tris-HCl. Heat inactivation was performed at 56°C for 30 minutes and the bacteria disrupted by sonication for 10' on ice (50% duty cycle, 50% output). Unbroken cells were removed by centrifugation at 5000g for 10 minutes and the total cell envelope

fraction recovered by centrifugation at 50000g at 4°C for 75 minutes. To extract cytoplasmic membrane proteins from the crude outer membranes, the whole fraction was resuspended in 2% sarkosyl (Sigma) and incubated at room temperature for 20 minutes. The suspension was centrifuged at 10000g for 10 minutes to remove aggregates, and the supernatant further ultracentrifuged at 50000g for 75 minutes to pellet the outer membranes. The outer membranes were resuspended in 10mM Tris-HCl, pH8 and the protein concentration measured by the Bio-Rad Protein assay, using BSA as a standard.

Whole Extracts preparation

Bacteria were grown overnight on a GC plate, harvested with a loop and resuspended in 1ml of 20mM Tris-HCl. Heat inactivation was performed at 56°C for 30' minutes.

Western blotting

Purified proteins (500ng/lane), outer membrane vesicles (5µg) and total cell extracts (25µg) derived from MenB strain 2996 were loaded onto a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The transfer was performed for 2 hours at 150mA at 4°C, using transfer buffer (0.3 % Tris base, 1.44 % glycine, 20% (v/v) methanol). The membrane was saturated by overnight incubation at 4°C in saturation buffer (10% skimmed milk, 0.1% Triton X100 in PBS). The membrane was washed twice with washing buffer (3% skimmed milk, 0.1% Triton X100 in PBS) and incubated for 2 hours at 37°C with mice sera diluted 1:200 in washing buffer. The membrane was washed twice and incubated for 90 minutes with a 1:2000 dilution of horseradish peroxidase labelled anti-mouse Ig. The membrane was washed twice with 0.1% Triton X100 in PBS and developed with the Opti-4CN Substrate Kit (Bio-Rad). The reaction was stopped by adding water.

Bactericidal assay

MC58 and 2996 strains were grown overnight at 37°C on chocolate agar plates. 5-7 colonies were collected and used to inoculate 7ml Mueller-Hinton broth. The suspension was incubated at 37°C on a nutator and let to grow until OD₆₂₀ was in between 0.5-0.8. The culture was aliquoted into sterile 1.5ml Eppendorf tubes and centrifuged for 20 minutes at maximum speed in a microfuge. The pellet was washed once in Gey's buffer (Gibco) and resuspended in the same buffer to an OD₆₂₀ of 0.5, diluted 1:20000 in Gey's buffer and stored at 25°C.

50µl of Gey's buffer/1% BSA was added to each well of a 96-well tissue culture plate. 25µl of diluted (1:100) mice sera (dilution buffer: Gey's buffer/0.2% BSA) were added to each well and the plate incubated at 4°C. 25µl of the previously described bacterial suspension were added to each well. 25µl of either heat-inactivated (56°C waterbath for 30 minutes) or normal baby rabbit complement were added to each well. Immediately after the addition of the baby rabbit complement, 22µl of each sample/well were plated on Mueller-Hinton agar plates (time 0). The 96-well plate was incubated for 1 hour at 37°C with rotation and then 22µl of each sample/well were plated on Mueller-Hinton agar plates (time 1). After overnight incubation the colonies corresponding to time 0 and time 1h were counted.

Gene Variability

The *ORF4* and *919* genes were amplified by PCR on chromosomal DNA extracted from various *Neisseria* strains (see list of strains). The following oligonucleotides used as PCR primers were designed in the upstream and downstream regions of the genes:

orf 4.1	(forward)	CGAATCCGGACGGCAGGACTC
orf 4.3	(reverse)	GGCAGGGAATGGCGGATTAAAG
919.1	(forward)	AAAATGCCTCTCCACGGCTG or CTGCGCCCTGTGTTAAAATCCCCT
919.6	(reverse)	CAAATAAGAAAGGAATTTTG or GGTATCGCAAACTTCGCCTTAATGCG

The PCR cycling conditions were:

1 cycle	2 min. at 94°
30 cycles	30 sec. at 94°
	30 sec. at ~ 54° or ~ 60° (in according to Tm of the primers)
	40 sec. at 72°
1 cycle	7 min. at 72°

The PCR products were purified from 1 % agarose gel and sequenced using the following primers:

orf 4.1 (forward)	CGAATCCGGACGGCAGGACTC
orf 4.2 (forward)	CGACCGCGCCTTTGGGACTG
orf 4.3 (reverse)	GGCAGGGAATGGCGGATTAAAG
orf 4.4 (reverse)	TCTTTGAGTTTGATCCAACC

- 919.1 (forward) AAAATGCCTCTCCACGGCTG or
CTGCGCCCTGTGTTAAAATCCCCCT
- 919.2 (forward) ATCCTTCCGCCTCGGCTGCG
- 919.3 (forward) AAAACAGCGGCACAATCGAC
- 919.4 (forward) ATAAGGGCTACCTCAAATC
- 919.5 (forward) GCGCGTGGATTATTTTGGG
- 919.6 (reverse) CAAATAAGAAAGGAATTTTG or
GGTATCGCAAACTTCGCCTTAATGCG
- 919.7 (reverse) CCCAAGGTAATGTAGTGCCG
- 919.8 (reverse) TAAAAAAAAGTTCGACAGGG
- 919.9 (reverse) CCGTCCGCCTGTCGTCGCCC
- 919.10 (reverse) TCGTTCCGGCGGGGTCGGGG

All documents cited herein are incorporated by reference in their entireties.

The following Examples are presented to illustrate, not limit, the invention

EXAMPLE 1

Using the above-described procedures, the following oligonucleotide primers were employed in the polymerase chain reaction (PCR) assay in order to clone the ORFs as indicated:

Table 1: Oligonucleotides used for PCR for Examples 2-10

ORF	Primer	Sequence	Restriction sites
279	Forward	CGCGGATCCCATATG-TTGCCTGCAATCACGATT <SEQ ID 3021>	BamHI-NdeI
	Reverse	CCCGCTCGAG-TTAGAAGCGGGCGGCAA <SEQ ID 3022>	XhoI
519	Forward	CGCGGATCCCATATG-TTCAAATCCTTTGTCGTCA <SEQ ID 3023>	BamHI-NdeI
	Reverse	CCCGCTCGAG-TTGCGGGTTTTGCTGC <SEQ ID 3024>	XhoI
576	Forward	CGCGGATCCCATATG-GCCGCCCCCGCATCT <SEQ ID 3025>	BamHI-NdeI
	Reverse	CCCGCTCGAG-ATTTACTTTTTGATGTCGAC <SEQ ID 3026>	XhoI
919	Forward	CGCGGATCCCATATG-TGCCAAAGCAAGAGCATC <SEQ ID 3027>	BamHI-NdeI
	Reverse	CCCGCTCGAG-CGGGCGGTATTCGGG <SEQ ID 3028>	XhoI
121	Forward	CGCGGATCCCATATG-GAAACACAGCTTTACAT <SEQ ID 3029>	BamHI-NdeI

128	Reverse	CCCGCTCGAG-ATAATAATATCCCGCGCCC <SEQ ID 3030>	XhoI
	Forward	CGCGGATCCCATATG-ACTGACAACGCACT <SEQ ID 3031>	BamHI-NdeI
206	Reverse	CCCGCTCGAG-GACCGCGTTGTCGAAA <SEQ ID 3032>	XhoI
	Forward	CGCGGATCCCATATG-AAACACCGCCAACCGA <SEQ ID 3033>	BamHI-NdeI
287	Reverse	CCCGCTCGAG-TTCTGTAAAAAAGTATGTGC <SEQ ID 3034>	XhoI
	Forward	CCGGAATTCTAGCTAGC-CTTTCAGCCTGCGGG <SEQ ID 3035>	EcoRI-NheI
406	Reverse	CCCGCTCGAG-ATCCTGCTCTTTTTGCC <SEQ ID 3036>	XhoI
	Forward	CGCGGATCCCATATG-TGCGGGACACTGACAG <SEQ ID 3037>	BamHI-NdeI
	Reverse	CCCGCTCGAG-AGGTTGTCCTTGTCTATG <SEQ ID 3038>	XhoI

Localization of the ORFs

The following DNA and amino acid sequences are identified by titles of the following form: [g, m, or a] [#].[seq or pep], where "g" means a sequence from *N. gonorrhoeae*, "m" means a sequence from *N. meningitidis B*, and "a" means a sequence from *N. meningitidis A*; "#" means the number of the sequence; "seq" means a DNA sequence, and "pep" means an amino acid sequence. For example, "g001.seq" refers to an *N. gonorrhoeae* DNA sequence, number 1. The presence of the suffix "-1" to these sequences indicates an additional sequence found for the same ORF, thus, data for an ORF having both an unsuffixed and a suffixed sequence designation applies to both such designated sequences. Further, open reading frames are identified as ORF #, where "#" means the number of the ORF, corresponding to the number of the sequence which encodes the ORF, and the ORF designations may be suffixed with ".ng" or ".a", indicating that the ORF corresponds to a *N. gonorrhoeae* sequence or a *N. meningitidis A* sequence, respectively. The word "partial" before a sequence indicates that the sequence may be a partial or a complete ORF. Computer analysis was performed for the comparisons that follow between "g", "m", and "a" peptide sequences; and therein the "pep" suffix is implied where not expressly stated. Further, in the event of a conflict between the text immediately preceding and describing which sequences are being compared, and the

279 gnm4.seq

m279.seq

m279.pep

g279.seq

g279 . pep

ORF 279 shows 89.5% identity over a 152 aa overlap with a predicted ORF (ORF 279.ng) from *N. gonorrhoeae*:

BNSDOCID: <WO__8957280A2_1_>

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1101 AGGTCGTCTG AAAGGCAGCT GGGGCGTGCA ATATTACAA CAAAAATCCA
1151 GTGCTTTATC TGCCATATCC GAAGCGGTTA AACAAACCGAT GCTGCTTGAC
1201 AACAAAGTGC AACATTACAG CTTTTTCGGT GTAGAACAGG CAAACTGGGA
1251 CAACTTCACG CTTGAAGGAG GCGTACGCGT GGA AAAACAA AAAGCCTCCA
1301 TTCAGTACGA CAAAGCATTG ATTGATCGGG AAAACTACTA CAACCAACCC
1351 CTGCCCCGACC TCGGCGCGCA CCGCCAAACC GCCCGCTCAT TCGCACTTTC
1401 GGGCAACTGG TATTTACACG CACAACACAA ACTCAGCCTG ACCGCCTCCC
1451 ATCAGGAACG CCTGCCGTCA ACGCAAGAGC TGTACGCACA CGGCAACAC
1501 GTCGCCACCA ACACCTTTGA AGTCGGCAAC AAACACCTCA ACAAGAGCG
1551 TTCCAACAAT ATCGAACTCG CGCTGGGCTA CGAAGGCGAC CGCTGGCAAT
1601 ACAATCTGGC ACTCTACCGC AACCGCTTCG GTA ACTACAT TTACGCCCAA
1651 ACCTTAAACG ACGGACGCGG CCCCAAATCC ATCGAAGACG ACAGCGAAAT
1701 GAAGCTCGTG CGCTACAACC AATCCGGCGC CGACTTCTAC GCGCGGAAG
1751 GCGAAATCTA CTTCAAACCG ACACCGCGCT ACCGCATCGG CGTTTCCGGC
1801 GACTATGTAC GAGGCCGTCT GAAAAACCTG CCTTCCCTAC CCGCAGAGA
1851 AGATGCCTAC GGCAACCGTC CTTTCATCGC ACAGGACGAC CAAATGCCCC
1901 CCCGTGTTCG GGCTGCGCGC CTCGGCTTCC ACCTGAAAGC CTCGCTGACC
1951 GACCGTATCG ATGCCAATTG GGACTACTAC CGCGTGTTCG CCCAAACAA
2001 ACTCGCCCGC TACGAAACGC GCACGCCCGG ACACCATATG CTCACCTCG
2051 GCGCAAATA CCGCCGCAAT ACGCGCTATG GCGAGTGGA TTGTACGTC
2101 AAAGCCGACA ACCTGCTCAA CCAATCCGTT TACGCCACA GCAGCTTCT
2151 CTCTGATACG CCGCAAATGG GCCGCAGCTT TACCGCGCGC GTGAACGTGA
2201 AGTTTAA

```

This corresponds to the amino acid sequence <SEQ ID 586; ORF 147>:

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m147.pep (partial)
1 ..PHKTEQSVDL ETVSVVGKSR PRATSGLLHT STASDKIISG DTLRQKAVNL
51 GDALDGVPGI HASQYGGGAS APVIRGQTGR RIKVLNHHGE TGDMAFSPD
101 HAIMVDTALS QQVEILRGPV TLLYSSGNVA GLVDVADGKI PEKMPENGVS
151 GELGLRLSSG NLEKLTSGGI NIGLKNFVL HTEGLYRKSG DYAVPRYRNL
201 KRLPDSHADS QTGSIGLSWV GEKGFIVGAY SDRRDQYGLP AHSHEYDDCH
251 ADIIWQKSLI NKRYLQLYPH LLTEEDIDYD NPGLSCGFHD DDNAHAHTS
301 GRPWIDLRNK RYELRAEWKQ PFPGFALRV HLNRRNDYRD EKAGDAVENF
351 FNNQTQNARI ELRHQPIGRL KGSWGVQYLQ QKSSALS AIS EAVKQFMLLD
401 NKVQHYSEFG VEQANWDNFT LEGGVRVEKQ KASIQYDKAL IDRENYNHP
451 LPDLGAHRQT ARSFALSGNW YFTPOHKLSL TASHQERLPS TQELYAHGKH
501 VATNTFEVGN KHLNKERSNN IELALGYEGD RWQYNLALYR NREFGNYIIAQ
551 TLNDGRGPKS IEDDSEMMLV RYNQSGADFY GAEGEYIFKP TPRYRIGVSG
601 DYVRGRKLNK PSLPGREDAY GNRPFIAQDD QNAPRVPAAR LGFHLKASLT
651 DRIDANLDYY RVFAQNKLAR YETRTPGHHM LNLGANYRRN TRYGEWNWYV
701 KADNLLNQSV YAHSSFLSDT PQMGRSFTGG VNVKF*

```

Computer analysis of this amino acid sequence gave the following results:
Homology with a predicted ORF from *N. gonorrhoeae*

m147 / g147 92.3% identity in 142 aa overlap

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                                10      20      30
m147.pep                      PHKTEQSVDL ETVSVVGKSR PRATSGLLHTS
                                |::|||  |||
g147      MRREAKMAQITLKPIVLSILLINTPLLAQAHETEQSVGLETVSVVGKSR PRATSGLLHTS
                                10      20      30      40      50      60

                                40      50      60      70      80      90
m147.pep      TASDKIISGDTLRQKAVNLGDALDGVPGI HASQYGGGASAPVIRGQTGRRIKVLNHHGET
                                |||
g147      TASDKIISGDTLRQKAVNLGDALDGVPGI HASQYGGGASAPVIRGQTGRRIKVLNHHGET
                                70      80      90      100     110     120

                                100     110     120     130     140     150
m147.pep      GDMADFS PDHAIMVDTALSQQVEILRGPV TLLYSSGNVAGLVDVADGKI PEKMPENGVS
                                |||
g147      GDMADFS PDHAIMVDTALSQQVEILRGPV TLLYSSGNVAGAGQCCRWNPPKNA

```

The following partial DNA sequence was identified in *N. meningitidis* <SEQ ID 587>:

a147.seq

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1 ATGCGACGAG AAGCCAAAT GGCACAACT AACTCAAAC CCATTGTTTT
51 ATCAATTCTT TTAATCAACA CACCCCTCCT CTCCAAGCG CATGGAACCTG
101 AGCAATCAGT GGGCTTGGAA ACGGTCAGCG TCGTCGGCAA AAGCCGTCGG
151 CGCGCCACTT CGGGGCTGCT GCACACTTCT ACCGCCTCCG ACAAATCAT
201 CAGCGGCGAC ACCTTGCGAC AAAAAGCCGT CAACTTGGGT GATGCTTTAG
251 ACGGCGTACC GGGCATTAT CATCTCGCAAT ACGGCGGCGG CGCATCCGCT
301 CCCGTTATTC GCGGTCAAAC AGGCAGACGG ATTAAAGTGT TGAACCATCA
351 CGGCGAAACG GGCACATGG CGGACTTCTC TCCAGACCAT GCAATCATGG
401 TGGACAGCGC CTGTGCGCAA CAGGTCGAAA TCCTGCGCGG TCCGGTTACG
451 CTCTTGATCA GCTCGGGCAA TGTGGCGGGG CTGGTCGATG TTGCCGATGG
501 CAAATCCCC GAAAAATGC CTGAAAACGG CGTATCGGGC GAACCTCGAT
551 TGCGTTTGAG CAGCGGCAAT CTGAAAAAAC TCACGTCGGG CGGCATCAAT
601 ATCGGTTTGG GCAAAACTT TGTATTGCAC ACGGAAGGGC TGTACCGCAA
651 ATCGGGGGAT TACGCCGTAC CGCGTTACCG CAATCTGAAA CGCCTGCCCG
701 ACAGCCACGC CGATTGCGAA ACGGGCAGCA TCGGGCTGTC TTGGGTTGGC
751 GAAAAAGGCT TTATCGGCGC AGCATACAGC GACCGTCGCG ACCAATATGG
801 TCTGCCTGCC CACAGCCACG AATACGATGA TTGCCACGCC GACATCATCT
851 GGCAAAAGAG TTTGATTAA ACACGCTATT TGCAGCTTTA TCCGCACCTG
901 TTGACCGAAG AAGACATCGA TTACGACAAT CCGGGCTTGA GCTGCGGCTT
951 TCACGACGAC GATGATGCAC ACGCCCATGC CCACAACGGC AAACCTTGGA
1001 TAGACCTGCG CAACAAACGC TACGAACTCC GCGCCGAATG GAAGCAACCG
1051 TTCCCGGGTT TTGAAGCCCT GCGCGTACAC CTGAACCGCA ACGACTACCG
1101 CCACGACGAA AAAGCAGGCG ATGCAGTAGA AAATTTTTT AACAACCAAA
1151 CGCAAAACGC CCGTATCGAG TTGCGCCACC AACCCATAGG CCGTCTGAAA
1201 GGCAGCTGGG CCGTGCAATA TTTGGGACAA AAATCCAGTG CTTTATCTGC
1251 CACATCCGAA GCGGTCAAAC AACCGATGCT GCTTGACAAT AAAGTGCAAC
1301 ATTACAGCTT TTTGCGTGT GAACAGGCAA ACTGGGACAA CTTACGCTT
1351 GAAGCGGCGG TACGCGTGGA AAAACAAAA GCCTCCATCC GCTACGACAA
1401 AGCATTGATT GATCGGGAAA ACTACTACAA CCATCCCCTG CCCGACCTCG
1451 GCGCGCACCG CCAAAACCGC CGCTCATTGC CACTTTCGGG CAACTGGTAT
1501 TTCAGCCAC AACACAACT CAGCCTGACC GCCTCCATC AGGAACGCTT
1551 GCCGTCAACG CAAGAGCTGT ACGCACACGG CAAACACGTC GCCACCAACA
1601 CCTTTGAAGT CGGCAACAAA CACCTCAACA AAGAGCGTTC CAACAATATC
1651 GAACTCGCGC TGGGCTACGA AGGCGACCGC TGGCAATACA ATCTGGCACT
1701 CTACCGCAAC CGCTTCGGCA ACTACATTTA CGCCCAAACC TTAAACGACG
1751 GACGCGGCCC CAAATCCATC GAAGACGACA GCGAAATGAA GCTCGTGGC
1801 TACAACCAAT CCGGTGCGGA CTTCTACGGC GCGGAAGGCG AAATCTACTT
1851 CAAACCGACA CCGCGTACC GCATCGGCGT TTCCGGCGAC TATGTACGAG
1901 GCGGCTGAAA AAACCTGCCT TCCCTACCGG GCAGGGAAGA CGCCTACGGC
1951 AACCGCCAC TCATTGCCA AGCCGACCAA AACGCCCTC GCGTTCCGGC
2001 TGCGCGCCTC GCGCTCCACC TGAAGCCTC GCTGACCGAC CGCATCGATG
2051 CCAATTGGA CTAATACCGC GTGTTGCGCC AAAACAACT CGCCCGCTAC
2101 GAAACGCGCA CGCCCGGACA CCATATGCTC AACCTCGGCG CAAACTACCG
2151 CCGCAATACG CGCTATGGCG AGTGGGAATTG GTACGTCAA GCGGACAACC
2201 TGCTCAACCA ATCCGTTTAC GCCACAGCA GCTTCCTCTC TGATACGCCG
2251 CAAATGGGCC GCAGCTTTAC CGGCGCGGTG AACGTGAAGT TTAA

```

This corresponds to the amino acid sequence <SEQ ID 588; ORF 147.a>:

a147.pep

```

1 MRREAKMAQT TLKPVLISIL LINTPLLSQA HGTEQSVGLE TVSVVGKSRP
51 RATSGLLHTS TASDKIISGD TLRQKAVNLG DALDGVPGIH ASQYGGGASA
101 PVIRGQTGRR IKVLNHHGET GDMADFSPDH AIMVDSALSQ QVEILRGPVT
151 LLYSSGNVAG LVDVADGKIP EKMPENGVSF ELGLRLSSGN LEKLTSGGIN
201 IGLGKNFVLH TEGLYRKSGD YAVPRYRNK RLPDSHADSQ TGSIGLSWVG
251 EKGFIGAAYS DRRDQYGLPA HSEYDDCHA DIIWQKSLIN KRYLQLYPHL
301 LTEEDIDYDN PGLSCGFHDD DDAHAHAHNG KPWIDLRNKR YELRAEWKQP
351 FPGFEALRVH LNRNDYRHDE KAGDAVENFF NNQTQNAIE LRHQPIGRK
401 GSWGVQYLGQ KSSALSATSE AVKQPMLLDN KVQHYSFVGV EQANWDNFTL
451 EGGVRVEKQK ASIRYDKALI DRENYNHPL PDLGAHRQTA RSFALSGNWI
501 FTPQHKLSTL ASHQERLPST QELYAHGKHV ATNTFEVGNK HLNKERSNNI
551 ELALGYEGDR WQYNLALYRN RFGNYIYAQT LNDGRGPKSI EDDSEMCLVR

```

415

601 YNQSGADFYG AEGEIYFKPT PRYRIGVSGD YVRGRKLNLP SLPGREDAYG
 651 NRPLIAQADQ NAPRVPAARL GVHLKASLTD RIDANLDYYR VFAQNKLARY
 701 ETRTPGHHML NLGANYRRNT RYGEWNWYVK ADNLLNQSVY AHSSFLSDTP
 751 QMGRSFTGGV NVKF*

m147/a147 98.1% identity in 734 aa overlap

m147.pep				10	20	30
				PHKTEQSV	DLTVSVVGKSR	PRATSGLLHTS
a147	MRREAKMAQTTLKPIVLSILLINTPLLSQA	HGTEQSVGLET	TVSVVGKSR	PRATSGLLHTS		
	10	20	30	40	50	60
m147.pep	40	50	60	70	80	90
	TASDKIISGDTLRQKAVNLGDALDGVPGI	HASQYGGGASAPVIRGQTGRRIKVLNHHGET				
a147	TASDKIISGDTLRQKAVNLGDALDGVPGI	HASQYGGGASAPVIRGQTGRRIKVLNHHGET				
	70	80	90	100	110	120
m147.pep	100	110	120	130	140	150
	GDMADFS	PDHAIMVDTALSQQVEILRGPVTL	LYSSGNVAGLVDVADGKIPEKMPENGVS			
a147	GDMADFS	PDHAIMVDSALSQQVEILRGPVTL	LYSSGNVAGLVDVADGKIPEKMPENGVS			
	130	140	150	160	170	180
m147.pep	160	170	180	190	200	210
	ELGLRLSSGNLEKLTSGGINIGLGNFVLHTEGLYRKSGDYAVPRYRN	KLRLPDSHAD				
a147	ELGLRLSSGNLEKLTSGGINIGLGNFVLHTEGLYRKSGDYAVPRYRN	KLRLPDSHAD				
	190	200	210	220	230	240
m147.pep	220	230	240	250	260	270
	TGSIGLSWVGKGF	IGVAYS	DRRDQYGLPAHSHEYDDCHADIIWQKSLINKRYLQLYPHL			
a147	TGSIGLSWVGKGF	IGAAYS	DRRDQYGLPAHSHEYDDCHADIIWQKSLINKRYLQLYPHL			
	250	260	270	280	290	300
m147.pep	280	290	300	310	320	330
	LTEEDIDYDN	PGLSCGFHDDDAHAH	THSGRPWIDL	RNCRYELRAEWKQFP	PGFEALRVH	
a147	LTEEDIDYDN	PGLSCGFHDDDAHAH	NGKPWDLRNCRYELRAEWKQFP	PGFEALRVH		
	310	320	330	340	350	360
m147.pep	340	350	360	370	380	390
	LNRNDYRHDEKAGDAVENFFN	QNTQNA	RIELRHQPIGR	LKGSWGVQYLQOKSSALS	SAISE	
a147	LNRNDYRHDEKAGDAVENFFN	QNTQNA	RIELRHQPIGR	LKGSWGVQYLQOKSSALS	SATSE	
	370	380	390	400	410	420
m147.pep	400	410	420	430	440	450
	AVKQPM	LLDNKVQHY	SFFGVEQANW	DNFTLEGGVRVEKQKAS	QYDKALIDRENYNHPL	
a147	AVKQPM	LLDNKVQHY	SFFGVEQANW	DNFTLEGGVRVEKQKAS	IRYDKALIDRENYNHPL	
	430	440	450	460	470	480
m147.pep	460	470	480	490	500	510
	PDLGAHRQTARS	FALSGN	WYFTPOHKLSLTASHQERLPSTQELYAHGKHVATNTFEVGNK			
a147	PDLGAHRQTARS	FALSGN	WYFTPOHKLSLTASHQERLPSTQELYAHGKHVATNTFEVGNK			
	490	500	510	520	530	540
m147.pep	520	530	540	550	560	570
	HLNKERSN	NIELALGYEGDRWQYNL	ALYRNRF	GNIIYAQTLNDGRGPKSIEDDSEMKLVR		
a147	HLNKERSN	NIELALGYEGDRWQYNL	ALYRNRF	GNIIYAQTLNDGRGPKSIEDDSEMKLVR		

416

	550	560	570	580	590	600
	580	590	600	610	620	630
m147.pep	YNQSGADFYGAEGEIIYFKPTPRYRIGVSGDYVRGRLKNLPSLPGREDAYGNRPFFIAQDDQ					
a147	YNQSGADFYGAEGEIIYFKPTPRYRIGVSGDYVRGRLKNLPSLPGREDAYGNRPLIAQADQ					
	610	620	630	640	650	660
	640	650	660	670	680	690
m147.pep	NAPRVPAARLGFHLKASLTDRIANLDYYRVFAQNKLARYETRTPGHHMLNLGANYRRNT					
a147	NAPRVPAARLGVHLKASLTDRIANLDYYRVFAQNKLARYETRTPGHHMLNLGANYRRNT					
	670	680	690	700	710	720
	700	710	720	730		
m147.pep	RYGEWNWYVKADNLLNQSVYAHSSFLSDTPQMGRSFTGGVNVKFX					
a147	RYGEWNWYVKADNLLNQSVYAHSSFLSDTPQMGRSFTGGVNVKFX					
	730	740	750	760		

The following partial DNA sequence was identified in *N. gonorrhoeae* <SEQ ID 589>:

```

g148.seq
1  ATGGCGTTAA  AAACATCAAA  CTTGGAACAC  GCAATGctgg  ttcaTCCCGA
51  AgctATgagt  gtcggcgCGC  TTGccgAcaa  AATCCGCAAA  AtcgaAAact
101 gGCCGCAAAA  AGgcaTCTTA  TTCCACGACA  TCACGCCCGT  CCTGCAAAGT
151 GCGGAATACT  TCCGCCTTTT  GGTGATTG  CTGGTTTACC  GCTATATGGA
201 TCAGAAAATC  GACATCGTTG  CCGGCTTGA  CGCGCGCGGC  TTCATTATCG
251 GCGCGGCACT  CGCCTACCAG  CTCaaCGtcg  gctTCGTCCC  CATCCGCAAA
301 AAAGGCAAGC  TGCCTTTTGA  AACCGTATCG  CAAAGCTAcg  cgcTCGAATA
351 CGGGGAAGCT  GCGGTGGAAA  TCCACACCGa  tgccgTCAAA  CCCGGTTCGC
401 GCGTCCTGCT  GGTGATGAT  TTGGTTGCCA  CGGGCGGCAC  AATGCTTGCC
451 GGGCTGGAAC  TGATCCGCAA  ACTCGGCGGG  GAAATTGTGCG  AAgccgcgcC
501 CATTTTGAA  TTTACCGACC  TTCAAGGCGG  CAAGAATATC  CGCGCAAGTG
551 GCGCGCCCTT  ATTTACCCTG  CTTCAAAACG  AAGGCTGCAT  GAAAGGCTGA

```

This corresponds to the amino acid sequence <SEQ ID 590; ORF 148.ng>:

```

g148.pep
1  MALKTSNLEH  AMLVHPEAMS  VGALADKIRK  IENWPQKGIL  FHDITPVLOS
51  AEYFRLLVDL  LVYRYMDQKI  DIVAGLDARG  FIIGAALAYQ  LNVGFVPIRK
101 KGKLPFETVS  QSYALEYGEA  AVEIHTDAVK  PGSRVLLVDD  LVATGGTMLA
151 GLELIRKLGG  EIVEAAALE  FTDLQGGKNI  RASGAPLFTL  LQNEGCMKG*

```

The following partial DNA sequence was identified in *N. meningitidis* <SEQ ID 591>:

```

m148.seq
1  ATGGCGTTAA  AAACATCAAA  CTTGGAACAC  GCAATGCTGG  TTCATCCCGA
51  AGCTATGAGT  GTCGGCGCGC  TTGCCGACAA  AATCCGCAAA  ATCGAAAAC
101 GGCCGCAAAA  AGGCATCTTA  TTCCACGACA  TCACGCCCGT  CCTTCAAAGC
151 GCGGAATACT  TCCGCCTTTT  GGTGATTG  TTGGTTTACC  GCTATATGGA
201 TCAGAAAATC  GACATCGTTG  CCGGTTTGA  CGCGCGCGGC  TTCATTATCG
251 GCGCGGCACT  CGCCTACCAG  CTCACGTCG  GTTTCGTCCC  CATCCGCAAA
301 AAAGGCAAGC  TGCCTTTTGA  AACCGTATCG  CAAAGCTACG  CGTCTGAATA
351 CGGGGAAGCT  GCGGTGGAAA  TCCACACCGA  TGCCGTCAAA  CTCGGTTCGC
401 GCGTCTGCT  GGTGATGAT  TTGATTGCCA  CGGGCGGCAC  GATGCTTGCC
451 GGAATGGAAC  TGATCCGCAA  ACTCGGCGGA  GAAATTGTGCG  AAGCCGCCCG
501 CATTTTGAA  TTTACCGACC  TTCAAGGCGG  CAAGAATATC  CGTGCAAGCG
551 GCGCGCCCTT  ATTTACCCTG  CTTCAAAACG  AAGGCTGTAT  GAAGGGCTGA

```

This corresponds to the amino acid sequence <SEQ ID 592; ORF 148>:

```

m148.pep
1  MALKTSNLEH  AMLVHPEAMS  VGALADKIRK  IENWPQKGIL  FHDITPVLOS
51  AEYFRLLVDL  LVYRYMDQKI  DIVAGLDARG  FIIGAALAYQ  LNVGFVPIRK

```

1396

```

|||||
g987  RKPPFIAGRLQDALKQPEKSVYLVSPYFVPTKSGTDALAKLVQDGDVTVLNSLQATDVA
      310      320      330      340      350      360

      370      380      390      400      410      420
m987.p AVHSGYVKYRKPLLKAGIKLYELQPNHAVPATKDKGLTGSSVTSLHAKTFIVDGKRIFIG
      |||||
g987  AVHSGYVKYRKPLLKAGIKLYELQPNHAVPATKDKGLTGSSVTSLHAKTFIVDGKRIFIG
      370      380      390      400      410      420

      430      440      450      460      470      480
m987.p SFNLDPRSARLNTMGVVIESPKIAEQMERTLADTTPAYAYRVTLDRHNRLQWHPATRK
      |||||
g987  SFNLDPRSARLNTMGVVIESPKIAEQMERTLADTTPAYAYRVTLDRHNRLQWHPATRK
      430      440      450      460      470      480

      490      500      509
m987.p TYPNEPEAKLWKRIAAILSLPIEGLLX
      |||||
g987  TYPNEPEAKLWKRIAAILSLPIEGLLX
      490      500

```

The following partial DNA sequence was identified in *N. meningitidis* <SEQ ID 2977>:

```

a987.seq
1  ATGAAACAC GCAGCCTAAT TTCCTTTTA TGCCTCCTC TCTGTTTCATG
51  TTCTTCATGG TTGCCCCAC TGAAGAACG GACGGAAAGC CGTCATTTC
101 ATACTTCAA ACCCGTCCG CTGGACAACA TCCTGCAAAT CCGGCACACC
151 CCTCATACCA ACGGGCTATC CGATATCTAT CTGTTGAACG ACCCCACGA
201 AGCCTTTGCC GCCCGCGCG CCCTTATCGA ATCTGCCGAA CACAGCCTCG
251 ATTGCAATA CTACATCTGG CGCAACGACA TTCCGGCCG ACTGCTGTT
301 AACCTCGTGT ACCTTGCCGC AGAACGCGGT GTGCGCGTAC GCCTGCTGT
351 GGACGACAAC AACACGCGCG GATTGGACGA CCTCCTGCTC GCCCTCGACA
401 GCCATCCCAA TATCGAAGTG CGCCTGTTC ACCCCTTCGT CTTACGAAAA
451 TGGCGCGCAC TCGGTACCT GACCGACTTC CCGCGCTCA ACCGCGCAT
501 GCACAACAAA TCCTTTACCG CCGACAACCG CGCCACCATA CTCGGCGGAC
551 GCAATATCGG CGACGAATAC TTCAAAGTCG GTGAGGACAC CGTTTTCGCC
601 GACCTGGACA TCCTCGCCAC CGGCAGCGTC GTCGGCGAAG TATCGCACGA
651 CTTGACCGCG TACTGGGCAA GCCATTCCGC CCACAACGCC ACGCGCATCA
701 TCCGCGCGCG CAACATCGGC AAGGGTCTTC AAGCACTCGG ATACAACGAC
751 GAAACGTCCA GACACGCGCT CCTGCGCTAC CGCGAAACCG TCGAACAGTC
801 GCCCTCTAC CAAAAATAC AGACAGGACG CATCGACTGG CAGAGCGTCC
851 AAACCCGCT CATCAGCGAC GACCCTGCAA AAGGACTCGA CCGCGACCG
901 CGCAAACCGC CGATTGCCG GCGGCTGCAA GACGCGCTCA AACAGCCCGA
951 AAAAAGCGTC TATCTGGTTT CACCCTATT CGTCCCACA AAATCCGGCA
1001 CAGACGCACT GGCAAACTG GTGAGGACG GCATAGACGT TACCGTCCTG
1051 ACCAACTCGC TACAGGCGAC CGACGTTGCC GCCGTCCATT CCGGCTATGT
1101 CAAATACCGA AAACCGCTGC TCAAAGCCG CATCAAACTC TACGAGCTGC
1151 AACCACAACCA TGCCGTCCCT GCCACAAAAG ACAAAGGCCT GACCGGCAGC
1201 TCCGTAACCA GCCTGCATGC CAAAACCTT ATTGTGGAG GCAAACGCAT
1251 CTTTCATCGG TCATTCAACC TCGACCCCGG TTCGCACGG CTCAATACTG
1301 AAATGGGCGT TGTATCGAA AGCCCCAAA TCGCAGAACA GATGGAGCGC
1351 ACCCTTGCCG ATACCTCACC CGAATACGCC TACCGCGTTA CCCTCGACAG
1401 GCACAACCGC CTGCAATGGC ACGATCCCGC CACCCGAAA ACCTACCCGA
1451 ACGAACCCGA AGCCAACTT TGGAAACGCA TCGCCGAAA AATCCTATCC
1501 CTGCTGCCCA TAGAAAGTT ATTATAG

```

This corresponds to the amino acid sequence <SEQ ID 550; ORF 2978.a>:

```

a987.pep
1  MKTRSLISLL CLLCSCSSW LPPLEERTES RHFNSTKPVRLDNILQIRHT
51  PHTNGLSDIY LLNDPHEAFA ARAALIESAE HSLDLQYYIW RNDISGRLLF
101 NLVYLAERG VRVRLLLDDN NTRGLDLLLL ALDHPNIEV RLFNPFVLRK
151 WRALGYLDF PRLNRRMHNK SFTADNRATI LGGRNIGDEY FKVGEDTVFA
201 DLDILATGSV VGEVSHDFDR YWASHAHNA TRIIRSGNIG KGLQALGYND
251 ETSRHALLRY RETVEQSPLY OKIQTGRIDW QSVQTRLISD DPAKGLDRDR
301 RKPPIAGRLQ DALQPEKSV YLVSPYFVPT KSGTDALAKL VQDGDVTVL

```

1397

351 TNSLQATDVA AVHSGYVKYR KPLLKAGIKL YELQPNHAVP ATKDKGLTGS
 401 SVTSLHAKTF IVDGKRIFIG SFNLDPRSAR LNTEMGVVIE SPKIAEQMER
 451 TLADTSPEYA YRVTLDRHNR LQWHPATRK TYPNEPEAKL WKRIAAKILS
 501 LPIESLL*

m987/a987 98.8% identity in 508 aa overlap

	10	20	30	40	50	60
m987.pep	MKTRSLISLLC	LLCSCSSWL	PPEERTES	RHFNTSKP	VRLDNILQ	IRHTPHTNGLSDIY
a987	MKTRSLISLLC	LLCSCSSWL	PPEERTES	RHFNTSKP	VRLDNILQ	IRHTPHTNGLSDIY
	70	80	90	100	110	120
m987.pep	LLNDPHEAFAA	RAALIESA	EHSLDLQY	YIWRNDIS	GRLLFNLV	LAAERGVRVRL
a987	LLNDPHEAFAA	RAALIESA	EHSLDLQY	YIWRNDIS	GRLLFNLV	LAAERGVRVRL
	130	140	150	160	170	180
m987.pep	NTRGLDDLL	LALD	SHPNIEV	RLENFV	LRKWRAL	GYLTDFPR
a987	NTRGLDDLL	LALD	SHPNIEV	RLENFV	LRKWRAL	GYLTDFPR
	190	200	210	220	230	240
m987.pep	LGGRNIGDEY	FKVGEDT	VFADLDIL	ATGSVVGE	VSHDFRY	WASHSAHN
a987	LGGRNIGDEY	FKVGEDT	VFADLDIL	ATGSVVGE	VSHDFRY	WASHSAHN
	250	260	270	280	290	300
m987.pep	KGLQALGYN	DETSRH	ALLRYRE	TVEQSPLY	QKIQTGC	IDWQSVR
a987	KGLQALGYN	DETSRH	ALLRYRE	TVEQSPLY	QKIQTGC	IDWQSVR
	310	320	330	340	350	360
m987.pep	RKPPIAGRL	QDALKQ	PEKSVYL	VSFVPTK	SGTDAL	AKLVQDG
a987	RKPPIAGRL	QDALKQ	PEKSVYL	VSFVPTK	SGTDAL	AKLVQDG
	370	380	390	400	410	420
m987.pep	AVHSGYVKYR	KPLLKAG	IKLYELQ	PNHAVP	ATKDKGL	TGSSVTSL
a987	AVHSGYVKYR	KPLLKAG	IKLYELQ	PNHAVP	ATKDKGL	TGSSVTSL
	430	440	450	460	470	480
m987.pep	SFNLDPRSAR	LNTEMG	VVIESPK	IAEQMER	TLADTTP	AYAYRV
a987	SFNLDPRSAR	LNTEMG	VVIESPK	IAEQMER	TLADTTP	AYAYRV
	490	500	509			
m987.pep	TYPNEPEAK	LWKRIA	AKILSLL	PIEGLLX		
a987	TYPNEPEAK	LWKRIA	AKILSLL	PIESLLX		

The following partial DNA sequence was identified in *N. gonorrhoeae* <SEQ ID 2979>:

g988.seq
 1 ATGAATAAAA ATATTAAATC TTAAATTTA CGGGAAAAAG ACCCGTTTTT

1419

```

m997      PLETASLRVLCNVLSGVLTKSGSDYLLPKQDLGAIVAEPALADLQRLGADIRLETRVC
           190      200      210      220      230      240

           250      260      270      280      290      300
a997.pep  RLNTLPDGKVLVNGEPFDAAVPATAPYHAAALLPEGTPEHVQTAYQNLRYHAITTVYLRY
           |||
m997      RLNTLPDGKVLVNGEAFDAAVPATAPYHAAALLPEGTPEHVQTAYQNLRYHAITTVYLRY
           250      260      270      280      290      300

           310      320      330      340      350      360
a997.pep  AEPVRLPAPLTGLADGTQVWLLCRGRLGLPENEVSAVISVSDRVGAFANRAWADKVHADL
           |||
m997      AEPVRLPAPLTGLADGTQVWLLCRGRLGLPENEVSAVISVSDRVGAFANRAWADKAHADL
           310      320      330      340      350      360

           370      380      390      400      410      420
a997.pep  KRILPHLGEPEAVRVITEKRATTAADAPPPDLSWLHRRHRIFFAGDYLHPDYPATLEAAVQ
           |||
m997      KRILPHLGEPEAVRVITEKRATTAADAPPPDLSWLHRRHRIFFAGDYLHPDYPATLEAAVQ
           370      380      390      400      410      420

           430
a997.pep  SGFASAEACLQSLSDAVX
           |||
m997      SGFASAEACLQSLSDAVX
           430

```

g999.seq Not found yet

a999.pep Not found yet

The following partial DNA sequence was identified in *N. meningitidis* <SEQ ID 3019>:

```

m999.seq
1  ATGAATATGA AAAAATTGAT TTCCGCAATT TGTGTTTCAA TTGTTTATC
51  AGCCTGCAAC CAACAATCAA AAACGGCACA AGCCGAAGAA CCTGTCCAAA
101 GTATCCAGGC TGCTGATTGT ACCGCCCCAA TGGACATCAC AGTTGAACAA
151 TATCTCATCA ATTTGGAGCA AGCATTTAAA ACTCAGAACG TCTCAACAAA
201 AATCCATAAT AAAAATATTG TCAAGACCGA TTGTGGTTAT GACCTTACTT
251 TGGTAATGGA TTTTGGGGCG ATTGCGCTCA AACTGGACGA GCAGCAAAAA
301 ATTAGAGCTA TCTCAGTAGG CTACATTTTA AAAACCGACG GAGAGAAAGG
351 ACAAAATCTA GTCAATAATG CCATAAATGG ATTACACAGT ATTCAGGCAG
401 TTCTGTCTTT AACTACCACA GACAAATTGG GCGAATCGGA AGCAGGAAAA
451 CAACTTTTTA CAGCTTTAAC CGAAGTCGTC AAAGAATCCA ATCAGACAGG
501 AGCAACAGCG CAAAAGACG TTCCGGCAGA TGGTATTTTA TATAGCGTTG
551 TTTTGTAAAA AGAAACAAAC ACCATTGCAA TAATCGGCAG AAAACAACCC
601 TAA

```

This corresponds to the amino acid sequence <SEQ ID 3020; ORF 999>:

```

m999.pep
1  MNMKKLISAI CVSIVLSACN QQSKTAQAEF PVQSIQAADC TAPMDITVEQ
51  YLINLEQAFK TQNVSTKIHN KNIVRTDCGY DLTIVMDFGA IALKLDEQQK
101 IRAISVGYIL KTDGEKGONL VNNAINGLHS IQAVLSLTIT DKLGESEAGK
151 QLFALTAEVV KESNQTGATA QKDV PADGIL YSVVFEKETN TIAIIGRKQP

```

a999.seq Not found yet

a999.pep Not found yet

The foregoing examples are intended to illustrate but not to limit the invention.

CLAIMS

1. A protein comprising a fragment of an amino acid sequence from SEQ ID 2790 wherein said fragment comprises at least 7 amino acids from said sequence.
2. A protein comprising an amino acid sequence selected from the group consisting of even numbered SEQ IDs from SEQ ID number 2 through SEQ ID number 3020.
3. A protein having 50% or greater homology to a protein according to claim 1.
4. A protein comprising a fragment of an amino acid sequence selected from the group consisting of even numbered SEQ IDs from SEQ ID number 2 through SEQ ID number 3020, wherein said fragment comprises 7 or more consecutive amino acids from said sequence.
5. An antibody which binds to a protein according to any one of claims 1 to 3.
6. A nucleic acid molecule which encodes a protein according to any one of claims 1 to 3.
7. A nucleic acid molecule according to claim 5, comprising a nucleotide sequence selected from the group consisting of odd numbered SEQ IDs from SEQ ID number 1 through SEQ ID number 3019.
8. A nucleic acid molecule comprising a fragment of a nucleotide sequence selected from the group consisting of odd numbered SEQ IDs from SEQ ID number 1 through SEQ ID number 3019, wherein said fragment comprises 10 or more consecutive nucleotides from said sequence.
9. A nucleic acid molecule comprising a nucleotide sequence complementary to a nucleic acid molecule according to claim 5.
10. A nucleic acid molecule comprising a nucleotide sequence complementary to a nucleic acid molecule according to claim 6.
11. A nucleic acid molecule comprising a nucleotide sequence complementary to a nucleic acid molecule according to claim 7.
12. A composition comprising a protein, a nucleic acid molecule, or an antibody according to any preceding claim.
13. A composition according to claim 11 being a vaccine composition or a diagnostic composition.
14. A composition according to claim 11 for use as a pharmaceutical.
15. The use of a composition according to claim 11 in the manufacture of a medicament for the treatment or prevention of infection due to Neisserial bacteria.

16. A composition comprising a protein of claim 1 wherein said composition is immunogenic.

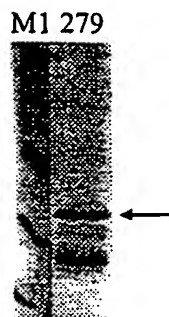
17. A composition comprising a protein of claim 2 wherein said composition is immunogenic.

18. A composition comprising a protein of claim 3 wherein said composition is immunogenic.

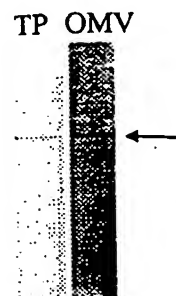
Fig. 2

279 (10.5 kDa)

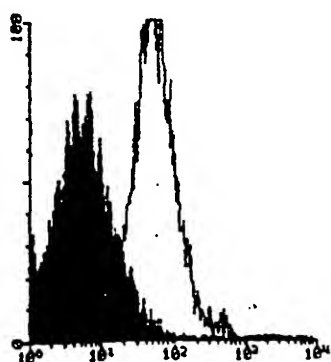
A) PURIFICATION



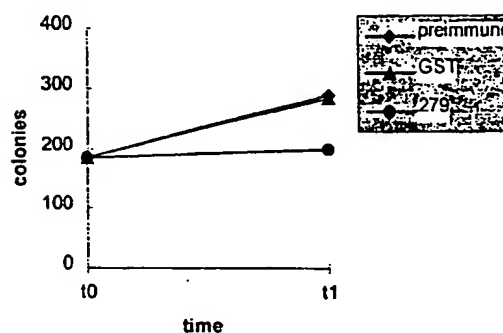
B) WESTERN BLOT



C) FACS



D) BACTERICIDAL ASSAY

E) ELISA assay: positive

279

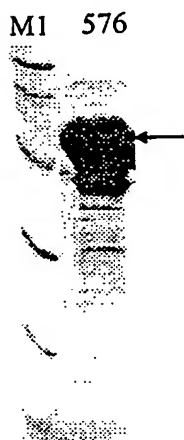
The predicted gene 279 was cloned in pGex vector and expressed in *E. coli*. The product of protein expression and purification was analyzed by SDS-PAGE. In panel A) is shown the analysis of 279-GST purification. Mice were immunized with the purified 279-GST and sera were used for Western blot analysis (panel B), FACS analysis (panel C), bactericidal assay (panel D), and ELISA assay (panel E). Results show that protein 279 is a surface-exposed protein. Symbols: M1, molecular weight marker; TP, *N. meningitidis* total protein extract; OMV, *N. meningitidis* outer membrane vesicle preparation. Arrows indicate the position of the main recombinant protein product (A) and the *N. meningitidis* immunoreactive band (B).

2/30

Fig. 3

576 (27.8 kDa)

A) PURIFICATION



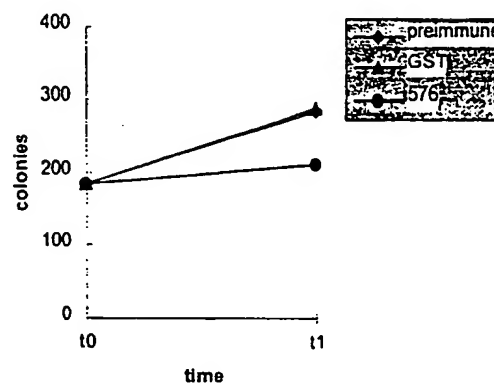
B) WESTERN BLOT



C) FACS



D) BACTERICIDAL ASSAY

E) ELISA assay: positive

576

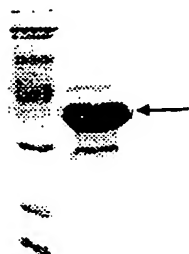
The predicted gene 576 was cloned in pGex vector and expressed in *E. coli*. The product of protein purification was analyzed by SDS-PAGE. In panel A) is shown the analysis of 576-GST fusion protein purification. Mice were immunized with the purified 576-GST and sera were used for Western blot (panel B), FACS analysis (panel C), bactericidal assay (panel D), and ELISA assay (panel E). Results show that 576 is a surface-exposed protein. Symbols: M1, molecular weight marker; TP, *N. meningitidis* total protein extract; OMV, *N. meningitidis* outer membrane vesicle preparation. Arrows indicate the position of the main recombinant protein product (A) and the *N. meningitidis* immunoreactive band (B).

Fig. 4

519 (33 kDa)

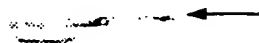
A) PURIFICATION

M1 519

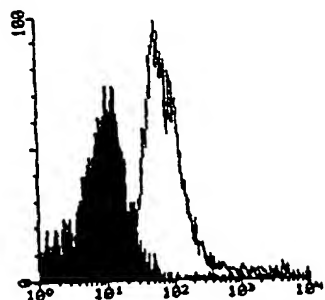


B) WESTERN BLOT

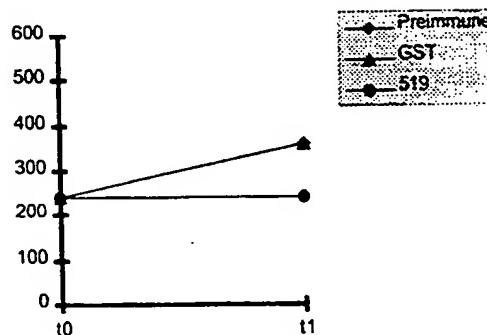
TP OMV



C) FACS



D) BACTERICIDAL ASSAY

E) ELISA assay: positive

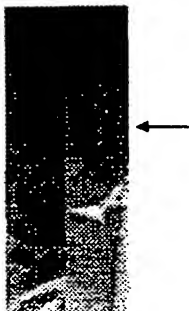
519

The predicted gene 519 was cloned in pET vector and expressed in *E. coli*. The product of protein purification was analyzed by SDS-PAGE. In panel A) is shown the analysis of 519-His fusion protein purification. Mice were immunized with the purified 519-His and sera were used for Western blot (panel B), FACS analysis (panel C), bactericidal assay (panel D), and ELISA assay (panel E). Results show that 519 is a surface-exposed protein. Symbols: M1, molecular weight marker; TP, *N. meningitidis* total protein extract; OMV, *N. meningitidis* outer membrane vesicle preparation. Arrows indicate the position of the main recombinant protein product (A) and the *N. meningitidis* immunoreactive band (B).

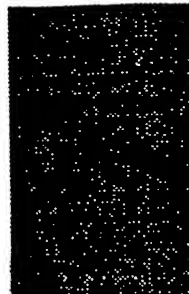
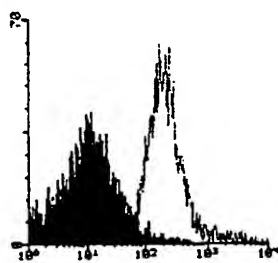
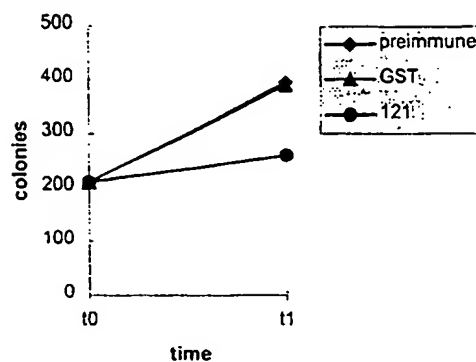
4/30

121 (40 kDa)**A) PURIFICATION**

M1 121

**B) WESTERN BLOT**

TP OMV

**C) FACS****D) BACTERICIDAL ASSAY****E) ELISA assay: positive****121**

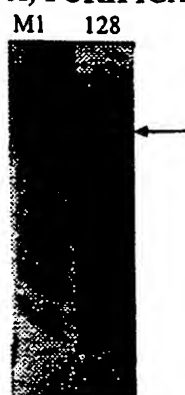
The predicted gene *121* was cloned in pET vector and expressed in *E. coli*. The product of protein purification was analyzed by SDS-PAGE. In panel A) is shown the analysis of 121-His fusion protein purification. Mice were immunized with the purified 121-His and sera were used for Western blot analysis (panel B), FACS analysis (panel C), bactericidal assay (panel D), and ELISA assay (panel E). Results show that 121 is a surface-exposed protein. Symbols: M1, molecular weight marker; TP, *N. meningitidis* total protein extract; OMV, *N. meningitidis* outer membrane vesicle preparation. Arrows indicate the position of the main recombinant protein product (A) and the *N. meningitidis* immunoreactive band (B).

Fig. 5

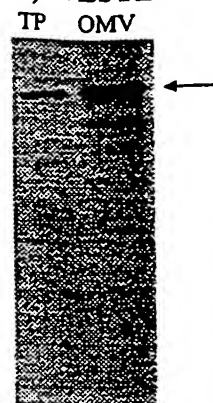
128 (101 kDa)

Fig. 6

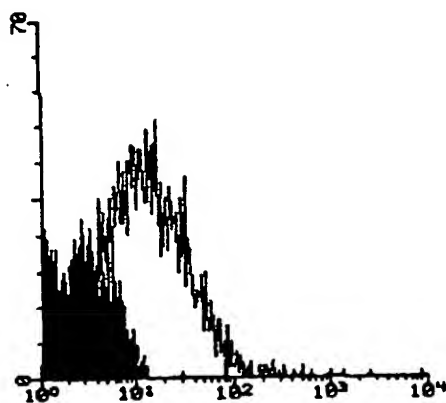
A) PURIFICATION



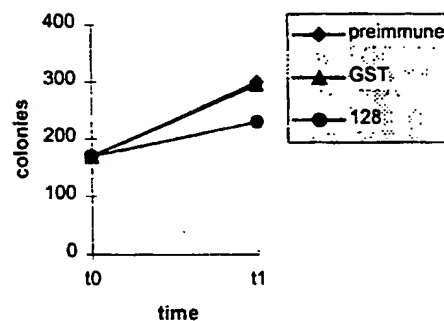
B) WESTERN BLOT



C) FACS



D) BACTERICIDAL ASSAY



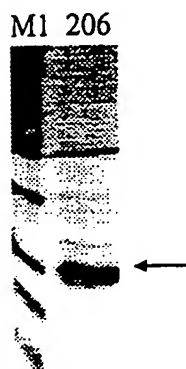
E) ELISA assay: positive

128

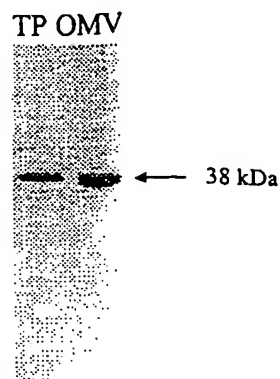
The predicted gene *128* was cloned in pET vector and expressed in *E. coli*. The product of protein purification was analyzed by SDS-PAGE. In panel A) is shown the analysis of 128-His purification. Mice were immunized with the purified 128-His and sera were used for Western blot analysis (panel B), FACS analysis (panel C), bactericidal assay (panel D) and ELISA assay (panel E). Results show that 128 is a surface-exposed protein. Symbols: M1, molecular weight marker; TP, *N. meningitidis* total protein extract; OMV, *N. meningitidis* outer membrane vesicle preparation. Arrows indicate the position of the main recombinant protein product (A) and the *N. meningitidis* immunoreactive band (B).

Fig. 7

A) PURIFICATION



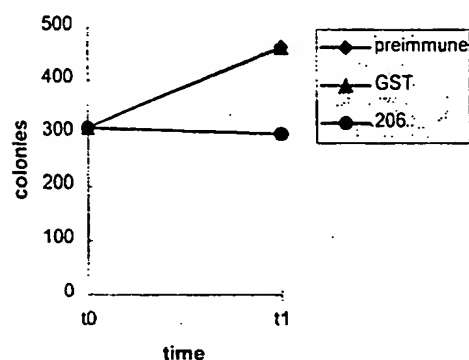
B) WESTERN BLOT



C) FACS



D) BACTERICIDAL ASSAY



E) ELISA assay: positive

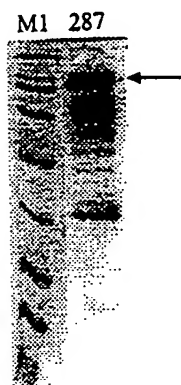
206

The predicted gene 206 was cloned in pET vector and expressed in *E. coli*. The product of protein purification was analyzed by SDS-PAGE. In panel A) is shown the analysis of 206-His purification. Mice were immunized with the purified 206-His and sera were used for Western blot analysis (panel B). It is worth noting that the immunoreactive band in protein extracts from meningococcus is 38 kDa instead of 17 kDa (panel A). To gain information on the nature of this antibody staining we expressed ORF 206 in *E. coli* without the His-tag and including the predicted leader peptide. Western blot analysis on total protein extracts from *E. coli* expressing this native form of the 206 protein showed a reactive band at a position of 38 kDa, as observed in meningococcus. We conclude that the 38 kDa band in panel B) is specific and that anti-206 antibodies, likely recognize a multimeric protein complex. In panel C) is shown the FACS analysis, in panel D) the bactericidal assay, and in panel E) the ELISA assay. Results show that 206 is a surface-exposed protein. Symbols: M1, molecular weight marker; TP, *N. meningitidis* total protein extract; OMV, *N. meningitidis* outer membrane vesicle preparation. Arrows indicate the position of the main recombinant protein product (A) and the *N. meningitidis* immunoreactive band (B).

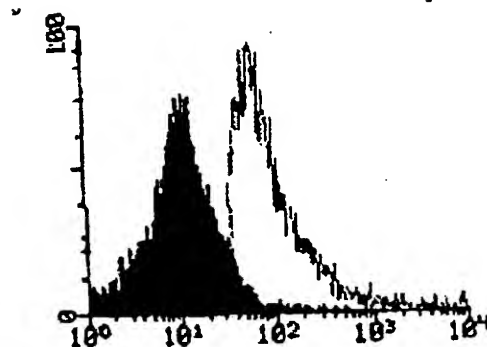
287 (78 kDa)

Fig. 8

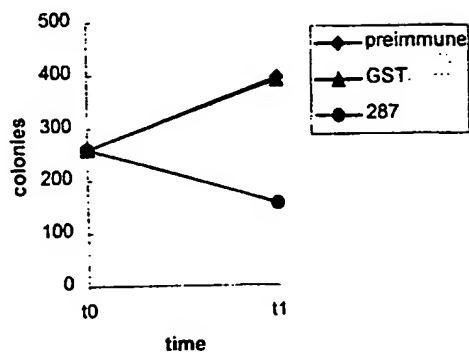
A) PURIFICATION



B) FACS



C) BACTERICIDAL ASSAY

D) ELISA assay : positive

287

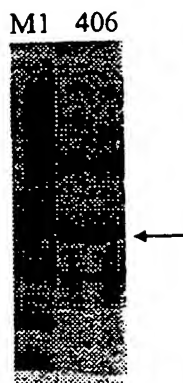
The predicted gene 287 was cloned in pGex vector and expressed in *E. coli*. The product of protein purification was analyzed by SDS-PAGE. In panel A) is shown the analysis of 287-GST fusion protein purification. Mice were immunized with the purified 287-GST and sera were used for FACS analysis (panel B), bactericidal assay (panel C), and ELISA assay (panel D). Results show that 287 is a surface-exposed protein. Symbols: M1, molecular weight marker. Arrow indicates the position of the main recombinant protein product (A).

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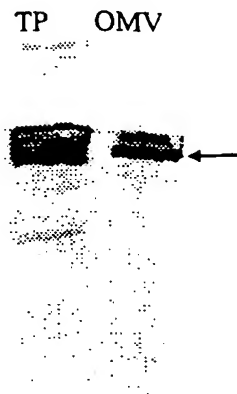
406 (33 kDa)

Fig. 9

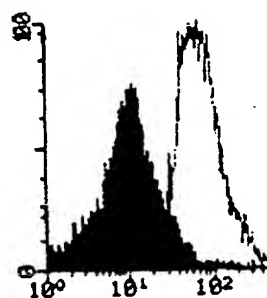
A) PURIFICATION



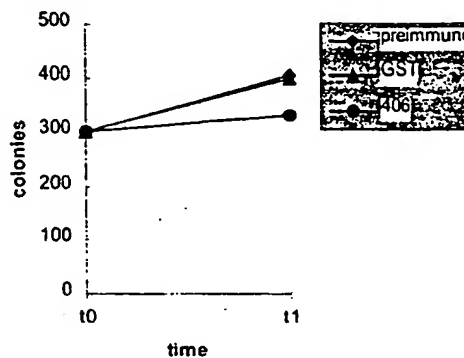
B) WESTERN BLOT



C) FACS



D) BACTERICIDAL ASSAY

E) ELISA assay : positive

406

The predicted gene *406* was cloned in pET vector and expressed in *E. coli*. The product of protein purification was analyzed by SDS-PAGE. In panel A) is shown the analysis of 406-His fusion protein purification. Mice were immunized with the purified 406-His and sera were used for Western blot analysis (panel B), FACS analysis (panel C), bactericidal assay (panel D), and ELISA assay (panel E). Results show that 406 is a surface-exposed protein. Symbols: M1, molecular weight marker; TP, *N. meningitidis* total protein extract; OMV, *N. meningitidis* outer membrane vesicle preparation. Arrows indicate the position of the main recombinant protein product (A) and the *N. meningitidis* immunoreactive band (B).

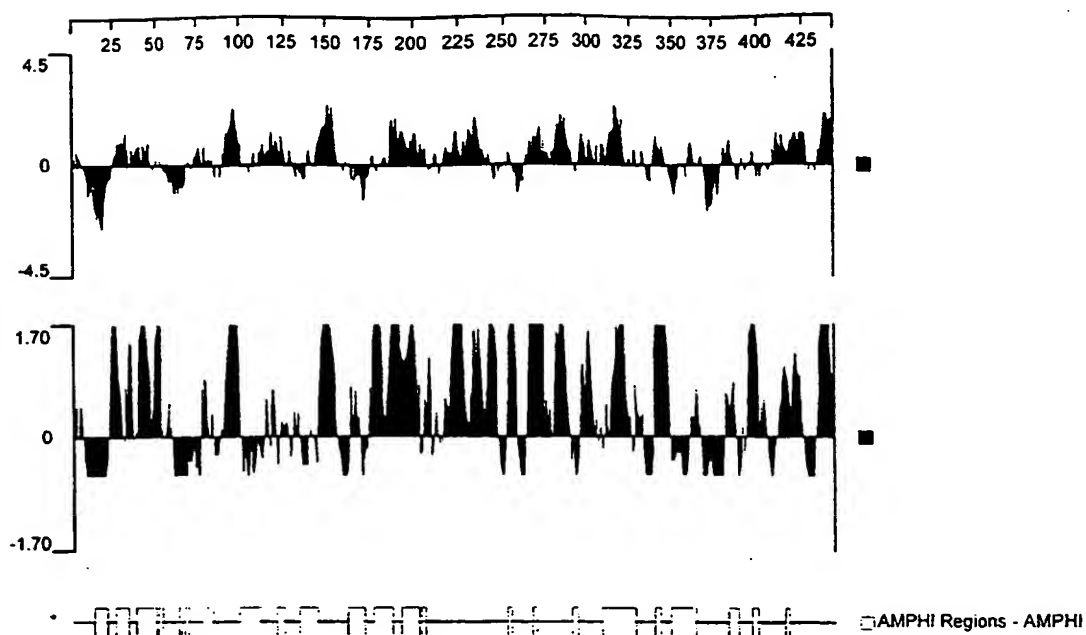
Hydrophilicity Plot, Antigenic Index and AMPHI Regions

Fig. 10

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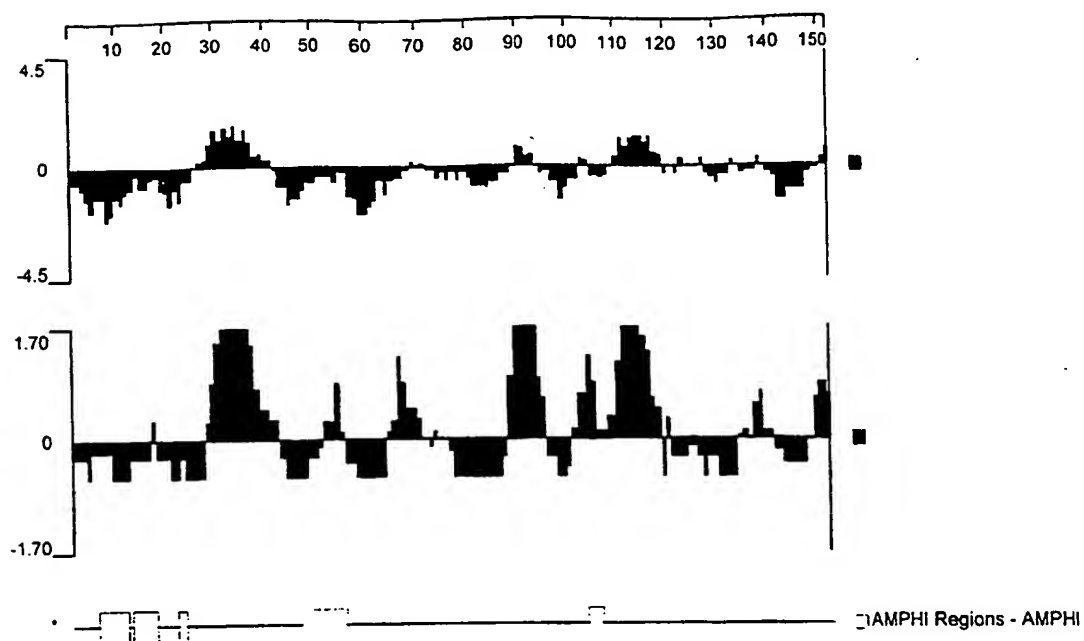
Hydrophilicity Plot, Antigenic Index and AMPHI Regions

Fig. 11

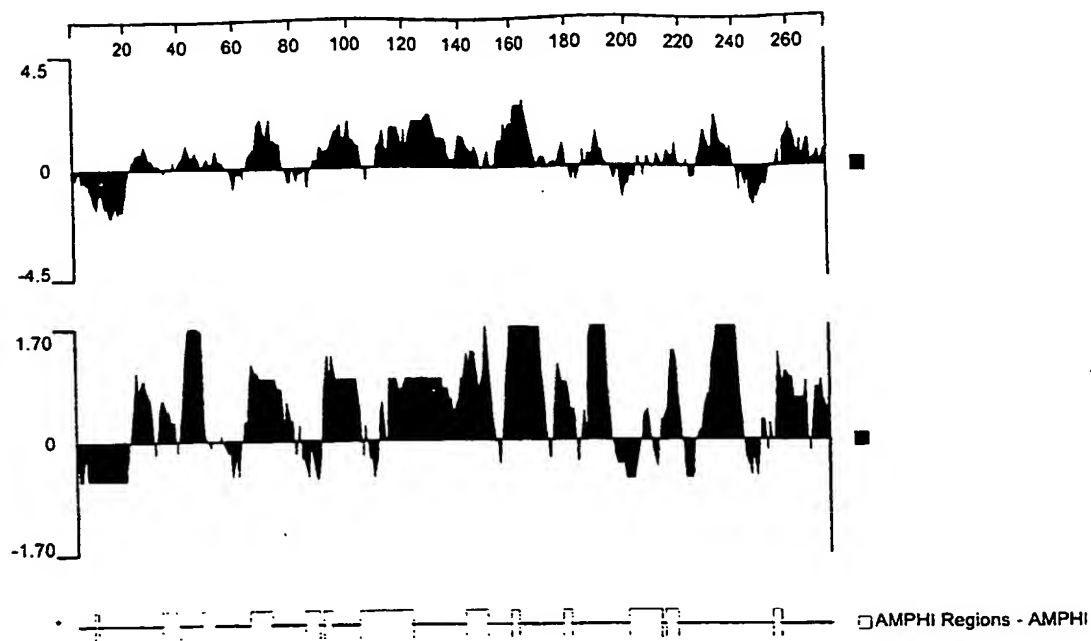
576-1Hydrophilicity Plot, Antigenic Index and AMPHI Regions

Fig. 12

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519-1

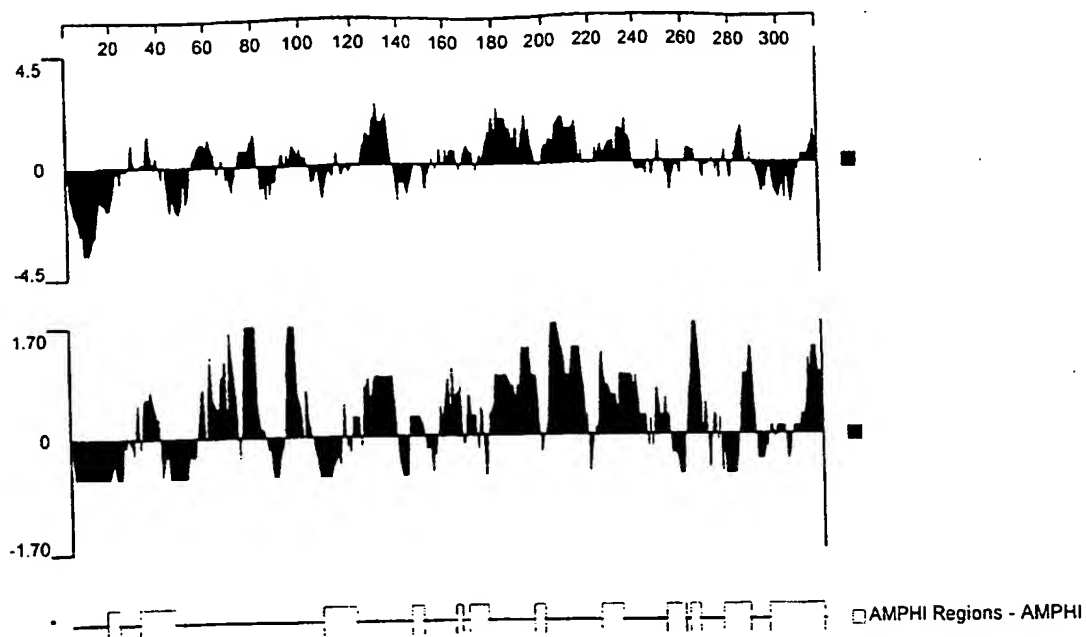
Hydrophilicity Plot, Antigenic Index and AMPHI Regions

Fig. 13

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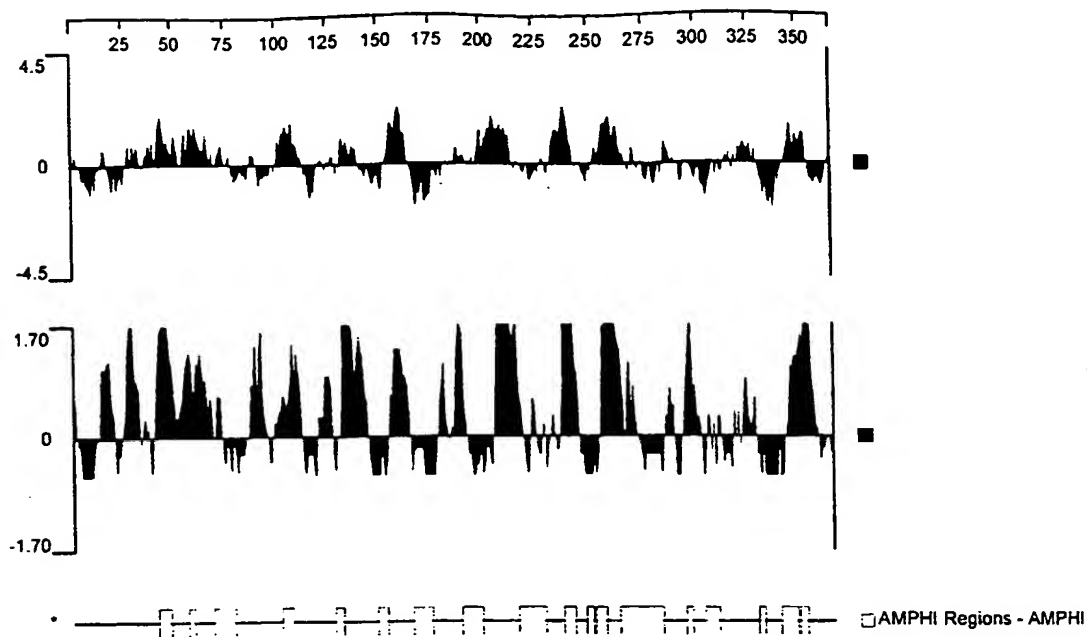
121-1Hydrophilicity Plot, Antigenic Index and AMPHI Regions

Fig. 14

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128-1

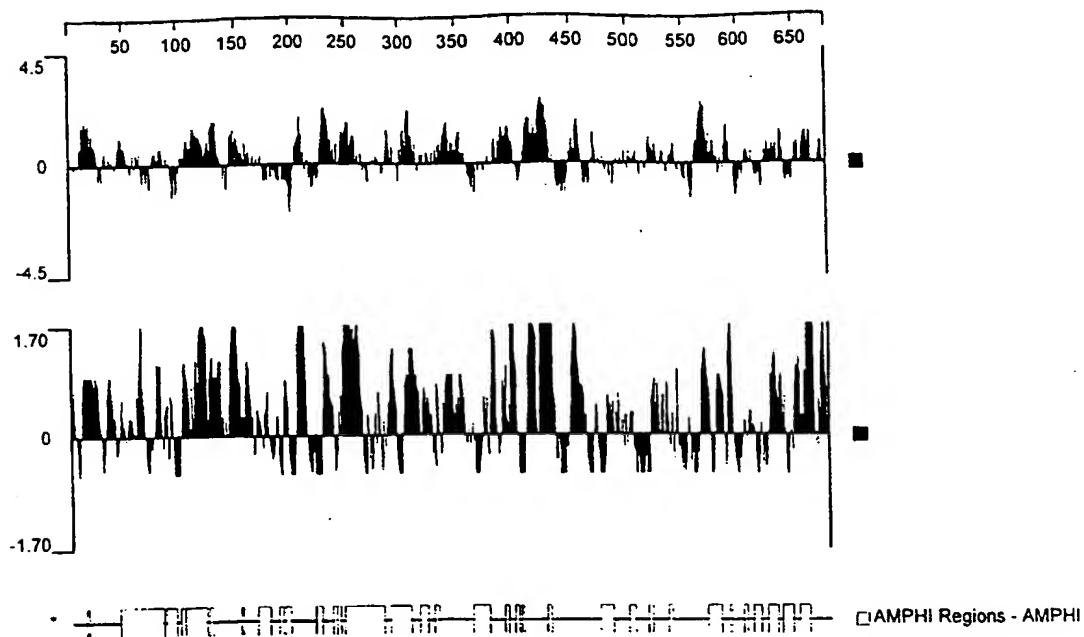
Hydrophilicity Plot, Antigenic Index and AMPHI Regions

Fig. 15

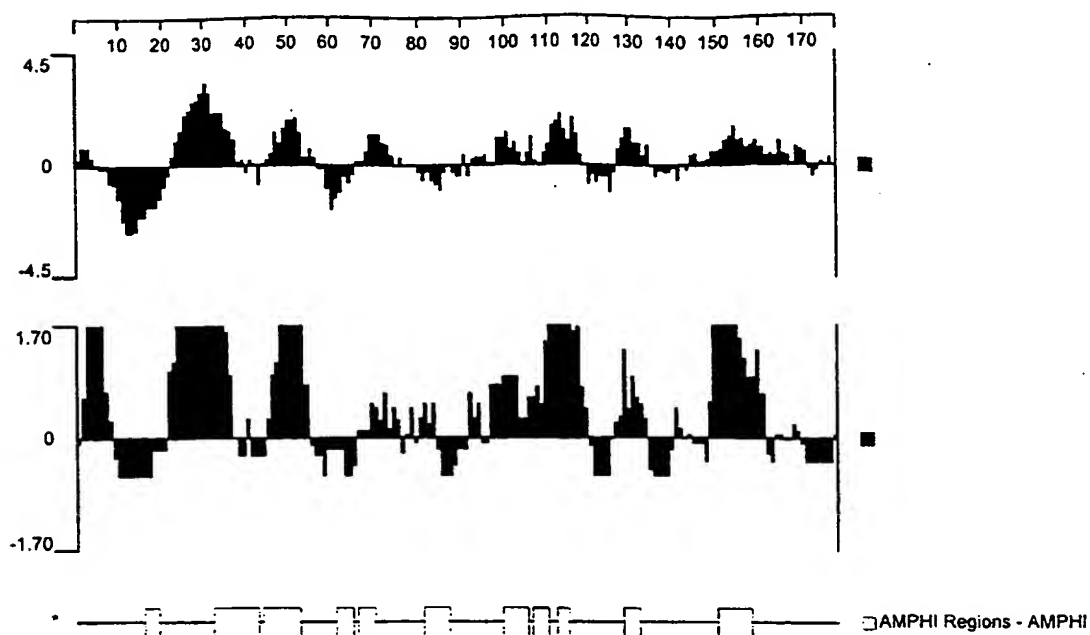
Hydrophilicity Plot, Antigenic Index and AMPHI Regions

Fig. 16

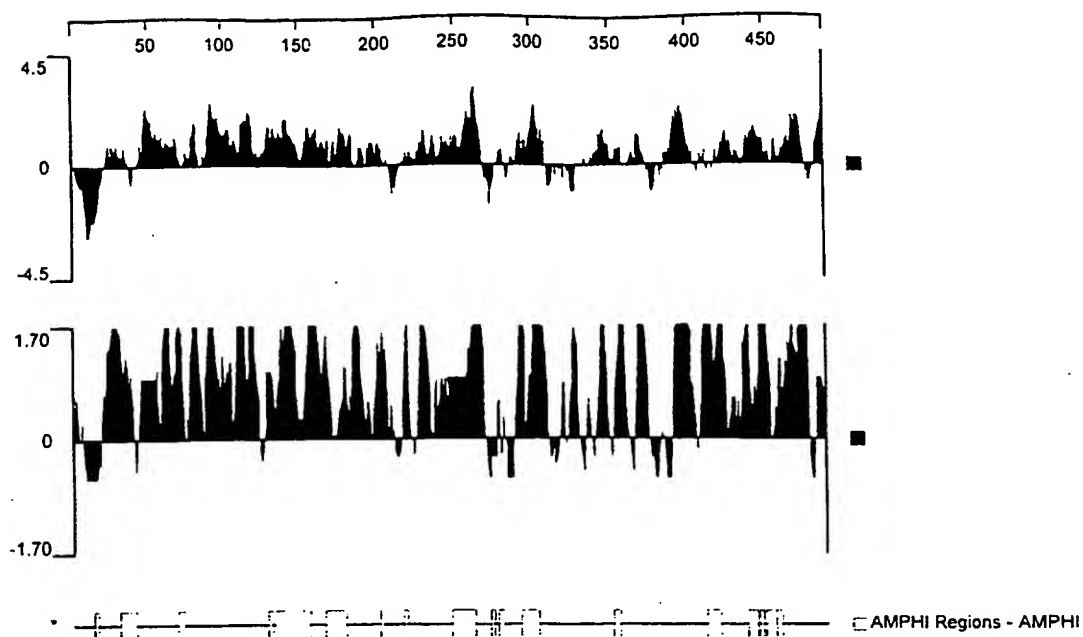
287Hydrophilicity Plot, Antigenic Index and AMPHI Regions

Fig. 17

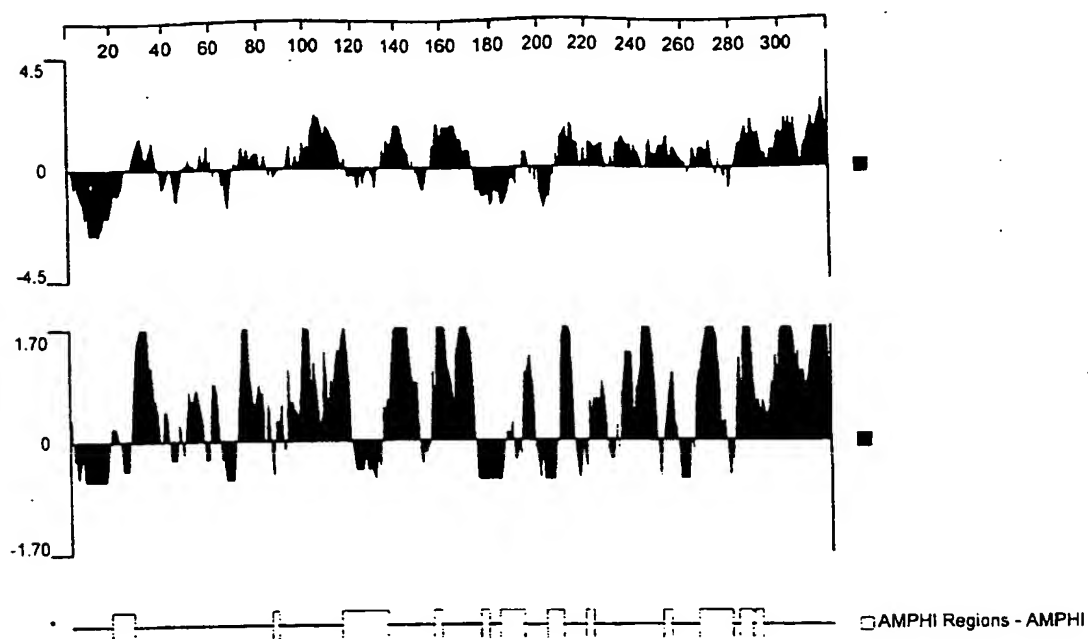
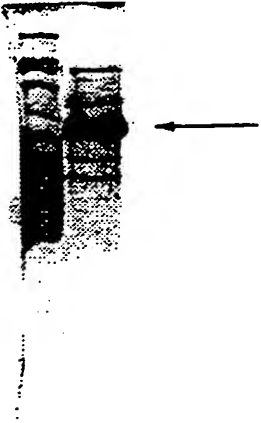
Hydrophilicity Plot, Antigenic Index and AMPHI Regions

Fig. 18

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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		(US). MASIGNANI, Vega [IT/IT]; Chiron S.p.A., Via Fiorentina, 1, I-53100 Siena (IT). MORA, Marirosa [IT/IT]; Chiron S.p.A., Via Fiorentina, 1, I-53100 Siena (IT). PETERSEN, Jeremy [US/US]; Arlington, VA (US). PIZZA, Mariagratia [IT/IT]; Chiron S.p.A., Via Fiorentina, 1, I-53100 Siena (IT). RAPPUOLI, Rino [IT/IT]; Chiron S.p.A., Via Fiorentina, 1, I-53100 Siena (IT). RATTI, Giulio [IT/IT]; Chiron S.p.A., Via Fiorentina, 1, I-53100 Siena (IT). SCALATO, Enzo [IT/IT]; Chiron S.p.A., Via Fiorentina, 1, I-53100 Siena (IT). SCARSELLI, Maria [IT/IT]; Chiron S.p.A., Via Fiorentina, 1, I-53100 Siena (IT). TETTELIN, Herve [US/US]; Gaithersburg, MD (US). VENTER, J., Craig [US/US]; Rockville, MD (US).	
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(57) Abstract <p>The invention provides proteins from <i>Neisseria meningitidis</i>, including the amino acid sequences and the corresponding nucleotide sequences. The proteins are predicted to be useful antigens for vaccines and/or diagnostics.</p>			
<div style="text-align: right;">919 (46 kDa) PURIFICATION M1 919 </div>			

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INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/31 C07K14/22 C07K16/12 C12Q1/68 A61K39/095 G01N33/50		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K C12Q A61K G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE TREMBL [Online] EMBL ID Q55666, AC Q55666, 1 November 1996 (1996-11-01) TABATA S: "Membrane-bound lytic transglycosylase A MltA Synechocystis sp. strain PCC 6803" XP002130156 Note: 100% aa seq identity of aa 342-350 with aa 392-400 of SEQ ID NOs 2790 and 2792, 27.6% (26.9%) aa seq identity with SEQ ID NO:2790 (2792) in 370 (387) aa overlap. the whole document --- -/--	1,4-6,9, 12
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C.		
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Date of the actual completion of the international search 26 May 2000		Date of mailing of the international search report 15.06.00
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl Fax: (+31-70) 340-3016		Authorized officer van de Kamp, M

11

INTERNATIONAL SEARCH REPORT

International Application No
PL./US 99/09346

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 818 465 A (BIOLOG MOLECULAIRE DES PLANTES ; INST OF MOLECULAR BIOTECHNOLOG (DE) 14 January 1998 (1998-01-14) Note: 100% nt seq identity of nt 367951-367961 of SEQ ID NO:1 with nt 163-173 of SEQ ID NO:2789. page 108	8,11,12
A	--- LOMMATZSCH J ET AL.: "Outer membrane localization of murein hydrolases: MltA, a third lipoprotein lytic transglycosylase in Escherichia coli" JOURNAL OF BACTERIOLOGY, vol. 179, no. 17, September 1997 (1997-09), pages 5465-5470, XP002130154 Note: 33.7% (35.7%) aa seq identity with SEQ ID NO:2790 (2792) in 273 (207) aa overlap. abstract	1-12
A	--- DILLARD J P ET AL.: "A peptidoglycan hydrolase similar to bacteriophage endolysins acts as an autolysin in Neisseria gonorrhoeae" MOLECULAR MICROBIOLOGY, vol. 25, no. 5, September 1997 (1997-09), pages 893-901, XP000878964 abstract	1-12
A	--- WO 96 29412 A (IAF BIO VAC INC ; BRODEUR BERNARD R (CA); MARTIN DENIS (CA); HAMEL) 26 September 1996 (1996-09-26) cited in the application the whole document examples 1-12	1-18
A	--- WO 94 08013 A (OREGON STATE) 14 April 1994 (1994-04-14) the whole document examples 1-7	1-18
A	--- WO 92 13871 A (UNIV WASHINGTON) 20 August 1992 (1992-08-20) the whole document examples 1-10	1-18
A	--- BLAKE M S ET AL.: "Vaccines for gonorrhoea: where are we on the curve?" TRENDS IN MICROBIOLOGY, vol. 3, no. 12, December 1995 (1995-12), pages 469-474, XP000876514 the whole document	1-18
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INTERNATIONAL SEARCH REPORT

International Application No

PL./US 99/09346

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	POOLMAN J T: "Development of a meningococcal vaccine" INFECTIOUS AGENTS AND DISEASE, vol. 4, no. 1, March 1995 (1995-03), pages 13-28, XP000876540 the whole document	1-18
X	WO 96 01901 A (RHONE POULENC RORER SA ;BLANC VERONIQUE (FR); THIBAUT DENIS (FR);) 25 January 1996 (1996-01-25) Note: 100% nt seq ident of bp 170-156 of SEQ ID NO:1 (rev DNA) with bp 202-216 of SEQ ID NO:1 (61.2% in 348 bp overlap), 40.7% seq ident of transl SEQ ID NO:1 with SEQ ID NO:2 in 118 aa overlap. page 102-104 example 1	8,11,12
X	WO 97 37044 A (ASTRA AB ;ALM RICHARD A (US); SMITH DOUGLAS (US)) 9 October 1997 (1997-10-09) Note: 100% aa seq identity of aa 204-211, 186-193 & 352-359 of transl SEQ ID NOs 227, 345 & 1003, resp., with aa 59-66 of SEQ ID NO:2, 37.4% aa seq identity with SEQ ID NO:2 in 115 aa overlap. page 268-269 page 344 page 909-910 page 23, paragraph B.4	4,12-14
X	DATABASE SWISSPROT [Online] ID YPCP YEREN, AC P31485, 1 July 1993 (1993-07-01) BAEUMLER A J ET AL.: "Hypothetical 29.6 kD protein in PCP 5' region (ORF1)" XP002138650 Note: 100% aa seq identity of aa 148-159 with aa 140-151 of SEQ ID NO:442, 43.4% aa seq identity with SEQ ID NO:442 in 256 aa overlap. the whole document	4,12
A	-& BAUMLER A J ET AL.: "A lipoprotein of Yersinia enterocolitica facilitates ferrioxamine uptake in Escherichia coli" JOURNAL OF BACTERIOLOGY, vol. 174, no. 3, February 1992 (1992-02), pages 1029-1035, XP000907295 page 1031, left-hand column, line 11 -right-hand column, line 15	4,12

INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	<p>DATABASE SWISSPROT [Online] ID YDHH HAEIN, AC P44861, 1 November 1995 (1995-11-01) FLEISCHMANN R D ET AL.: "Hypothetical protein HI0753" XP002138651 Note: 100% aa seq identity of aa 143-156 with aa 140-153 of SEQ ID NO:442, 41.6% aa seq identity with SEQ ID NO:442 in 377 aa overlap. the whole document</p>	4,12
X	<p>WO 96 33276 A (HUMAN GENOME SCIENCES INC ;UNIV JOHNS HOPKINS (US)) 24 October 1996 (1996-10-24) Note: 100% nt seq identity of bp 816794-816807 with bp 289-302 of SEQ ID NO:441 (54.3% in 484 bp overlap), 100% aa seq identity of translated sequence with SEQ ID NO:442 in 14 aa overlap. page 77.488 Note: 100% nt seq identity of bp 230516-230526 with bp 1501-1511 of SEQ ID NO:489 (57.4% in 1292 bp overlap), 100% aa seq identity of translated sequence with SEQ ID NO:490 in 13 aa overlap. page 77.139 page 76.37, line HI0215 Note: 100% nt seq identity of bp 1025409-1025418 with bp 1339-1330 (rev strand) of SEQ ID NO:1201 (72.0% in 50 bp overlap). page 77.612</p>	4,8, 11-14
X	<p>CONLIN C A ET AL.: "Escherichia coli prlC encodes an endopeptidase and is homologous to the Salmonella typhimurium opdA gene" JOURNAL OF BACTERIOLOGY, vol. 174, no. 18, September 1992 (1992-09), pages 5881-5997, XP000907300 Note: 100% nt seq ident of bp 1824-1837 with bp 1480-1493 of SEQ ID NO:489 (59.7% in 1282 bp overlap), 100% aa seq ident of aa 495-507 with aa 492-504 of SEQ ID NO:490 (49.5% in 679 aa overlap). abstract figure 2</p>	4,8,11, 12

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE SWISSPROT [Online] ID OPDA_HAEIN, AC P44573, 1 November 1995 (1995-11-01) FLEISCHMANN R D ET AL.: "Oligopeptidase A (EC 3.4.24.70)" XP002138652 Note: 100% aa seq identity of aa 496-508 with aa 492-504 of SEQ ID NO:490, 49.0% aa seq identity in 677 aa overlap. the whole document</p>	4,12
X	<p>--- ROKBI B ET AL.: "Evaluation of recombinant transferrin - binding protein B variants from Neisseria meningitidis for their ability to induce cross-reactive and bactericidal antibodies against a genetically diverse collection of serogroup B strains." INFECTION AND IMMUNITY, vol. 65, no. 1, January 1997 (1997-01), pages 55-63, XP002138643 abstract</p>	5
P,A	<p>--- DATABASE TREMBL [Online] EMBL ID 069750, AC 069750, 1 August 1998 (1998-08-01) ROKBI B ET AL.: "Transferrin binding protein B, TbpB, Neisseria meningitidis" XP002138653 Note: 22.3% aa seq identity with SEQ ID NO:1202 in 488 aa overlap. the whole document</p>	4,8, 12-15,17
A	<p>-& ROKBI B ET AL.: "Heterogeneity of tbpB, the transferrin-binding protein B gene, among serogroup B Neisseria meningitidis strains of the ET-5 complex" CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol. 4, no. 5, September 1997 (1997-09), pages 522-529, XP002138644 abstract</p> <p>--- -/--</p>	5,8, 12-15,17

INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DATABASE GCG GENESSEQ [Online] ID W14640, AC W14640, 5 March 1998 (1998-03-05) QUENTIN-MILLET M J ET AL.: "N. meningitidis HTR Tbp2 (del377-385, del407-465, del488-508)" XP002138654 Note: 23.5% aa seq identity with SEQ ID NO:1202 in 571 aa overlap. the whole document</p>	4,8, 12-15,17
A	<p>-& WO 97 13860 A (PASTEUR MERIEUX SERUMS VACC; QUENTIN MILLET MARIE JOSE (FR); ROKBI)) 17 April 1997 (1997-04-17) claim 11</p>	4,8, 12-15,17
X	<p>--- DATABASE EMPRO1 [Online] EMBL ID AF034831, AC AF034831, 4 December 1997 (1997-12-04) YOU Z ET AL.: "Rhizobium etli stomatin like protein (slp) gene, complete cds." XP002138655 Note: 100% nt seq ident of bp 4384-4395 with bp 529-540 of SEQ ID NO:1455 (54.4% in 638 bp overlap), 100% aa seq ident of aa 1394-1403 with aa 109-118 of SEQ ID NO:1456 (41.2% in 182 aa overlap). the whole document</p>	4,8,11, 12
P,X	<p>-& YOU Z ET AL.: "A stomatin-like protein encoded by the slp gene of Rhizobium etli is required for nodulation competitiveness on the common bean" MICROBIOLOGY, vol. 144, no. 9, September 1998 (1998-09), pages 2619-2627, XP000907294 abstract figure 2</p>	4,8,11, 12
X	<p>--- HUANG M ET AL.: "A stomatin-like protein necessary for mechanosensation in C. elegans" NATURE, vol. 378, no. 6554, 16 November 1995 (1995-11-16), pages 292-295, XP002138646 Note: 100% aa seq identity of aa 233-239 with aa 110-117 of SEQ ID NO:1456, 29.9% aa seq identity in 234 aa overlap. abstract figure 1</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	4,12

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WONG C Y ET AL.: "Cloning and characterization of two immunophilin-like genes, ilpA and fkpA, on a single 3.9-kilobase fragment of Aeromonas hydrophila genomic DNA"</p> <p>JOURNAL OF BACTERIOLOGY, vol. 179, no. 11, June 1997 (1997-06), pages 3397-3403, XP002138647</p> <p>Note: 100% nt seq ident of bp 2659-2672 with bp 613-626 of SEQ ID NO:1745 (59.2% in 655 bp overlap), 100% aa seq ident of aa 205-216 with aa 200-211 of SEQ ID NO:1746 (44.9% in 265 aa overlap).</p> <p>abstract figure 2</p>	4,8, 11-14
X	<p style="text-align: center;">---</p> <p>DATABASE EMPRO2 [Online]</p> <p>EMBL</p> <p>ID NE01198, AC U001198, 23 November 1993 (1993-11-23)</p> <p>MCALLISTER C F ET AL.: "Neisseria elongata NRL FKBP immunophilin homolog gene"</p> <p>XP002138656</p> <p>Note: 100% nt seq identity of bp 125-138 with bp 635-648 of SEQ ID NO:1745 (65.8% nt seq identity in 237 bp overlap).</p> <p>the whole document</p>	8,11,12
X	<p>-& MCALLISTER C F ET AL.: "Analysis in Neisseria meningitidis and other Neisseria species homologous to the FKBP immunophilin family"</p> <p>MOLECULAR MICROBIOLOGY, vol. 10, no. 1, October 1993 (1993-10), pages 13-23, XP000907304</p> <p>abstract figure 3</p>	8,11,12
X	<p style="text-align: center;">---</p> <p>SAMPSON B A ET AL.: "Neisseria meningitidis encodes an FK506-inhibitable rotamase"</p> <p>PROC. NAT'L. ACAD. SCI. USA, vol. 89, no. 4, 15 February 1992 (1992-02-15), pages 1164-1168, XP002138648</p> <p>Note: 100% nt seq identity of bp 278-288 (284-294) with bp 719-729 of SEQ ID NO:1745 (60.5% nt seq identity in 281 bp overlap).</p> <p>abstract figure 2</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	8,11,12

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International Application No
PL./US 99/09346

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HACKER J ET AL.: "Immunophilins: structure-function relationship and possible role in microbial pathogenicity." MOLECULAR MICROBIOLOGY, vol. 10, no. 3, November 1993 (1993-11), pages 445-456, XP000907321 abstract	13,14,17
X	--- DATABASE EMPRO1 [Online] EMBL ID ECUW93, AC U14003 (partial), 30 November 1994 (1994-11-30) BURLAND V ET AL.: "Escherichia coli K-12 chromosomal region from 92.8 to 00.1 minutes" XP002138657 Note: 100% nt seq identity of bp 37827-37839 with bp 1186-1174 of SEQ ID NO:2791. page 4 -----	8,11,12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/09346

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
1,3,16,18 (all completely); 2,4-15,17 (all partially). Inventions searched:
#1 (SEQ ID NOs 2789/2790), #2 (1/2), #222 (441/442), #246 (489/490), #602 (1201/1202), #729 (1455/1456), #874 (1745/1746), #1397 (2791/2792)
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention 1. Claims: 1,3,16,18 (all completely); 2,4-15,17 (all partially)

A protein comprising the amino sequence of SEQ ID NO:2790 or comprising a fragment of at least 7 (preferably consecutive) amino acids of said SEQ ID NO; a protein with 50% or greater homology to said protein(s); an antibody binding to said protein(s); a nucleic acid encoding said protein(s), preferably comprising the nucleotide sequence of SEQ ID NO:2789 or a fragment comprising 10 or more consecutive nucleotides thereof; complementary nucleic acid molecules; compositions comprising said protein(s), nucleic acid(s) or antibody for vaccination, diagnosis or pharmaceutical use, preferably immunogenic compositions comprising said protein(s), and the use of said composition(s).

Invention 2. Claims: 2,4-15,17 (all partially)

A protein comprising an amino sequence according to SEQ ID NO:2 or comprising a fragment of at least 7 consecutive amino acids of said SEQ ID NO; an antibody binding to said protein(s); a nucleic acid encoding said protein(s), preferably comprising a nucleotide sequence according to SEQ ID NO:1 or a fragment comprising 10 or more consecutive nucleotides thereof; complementary nucleic acid molecules; compositions comprising said protein(s), nucleic acid(s) or antibody for vaccination, diagnosis or pharmaceutical use, preferably immunogenic compositions comprising said protein(s), and the use of said composition(s).

Inventions 3-1510. Claims: 2,4,-15,17 (all partially)

Same as invention 2 but for proteins limited to the even-numbered SEQ ID NOs:4-3020 except 2790, and for nucleic acids limited to the corresponding odd-numbered SEQ ID NOs:3-3019 except 2789. E.g., invention 3: limited to SEQ ID NO:4 and SEQ ID NO:3, invention 4: limited to SEQ ID NO:6 and SEQ ID NO:5, ... , invention 1509: limited to SEQ ID NO:3018 and SEQ ID NO:3017, and invention 1510: limited to SEQ ID NO:3020 and SEQ ID NO:3019.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

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